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10

Pharmacology and Histology of the Therapeutic Application of Botulinum Toxin

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INTRODUCTION

Since the introduction of botulinum toxin (BTX) into therapeutics in 1978 for strabismus (1–3), its use has been expanded to other indications including blepharospasm, adult-onset spasmodic torticollis, spasmodic dysphonia, occupational hand cramps, and jaw dystonia. Application of this therapy to other disorders is on the horizon and is contributing to the driving force for expansion of clinical and basic research (4–12). However, despite the success obtained with BTX for treatment of blepharospasm and other focal and segmental movement disorders, its application is limited by the following properties of the therapy:

1. Need for indefinitely repeating injections when treating chronic disease
2. Untoward spread of toxin to other muscles not targeted for injection
3. Antibody formation with resistance to the therapeutic action of the toxin after repeated injections
4. The consistency of biologic activity contained within the labeled vials
5. Lack of standardization of the injections sites
6. Placement of the therapeutic toxin preparation into the correct anatomical position when access to the muscles is difficult, requiring electromyographic assistance (particularly for treatment of occupational hand disorders)
7. Inadequate understanding of long-term benefits and effects of repeated treatment with the therapeutic preparation
8. Inadequate comprehension of the nature of any permanent microanatomical changes in striated muscle and motor nerve terminals after repeated botulinum toxin injections

9. Inadequate fundamental understanding of the pathophysiology of focal and segmental dystonias

This chapter will address the histologic changes produced by therapeutic injections of botulinum toxin with pertinent clinical data, so that clinical-pathologic correlations can be made. Some of the issues outlined above will be explored.

BIOCHEMICAL CHARACTER AND HISTOLOGIC EFFECTS OF BOTULINUM TOXIN IN STRIATED MUSCLE

Biochemistry and Cellular Physiology

The first apparent histologic effect appears to be clustering of vesicles at the presynaptic membrane, indicating an impairment of release of acetylcholine (13). This finding has been well documented with electron microscopy (13).

The therapeutic actions of BTX are a consequence of the complex structure and function of this molecule and its actions at the nerve terminal. The BTX molecule has a molecular weight of approximately 150,000 daltons, and the active form of the toxin exists as a dichain molecule consisting of a light (M_r approximately 50,000 daltons) and heavy (M_r approximately 100,000 daltons) chain linked by a disulfide bond (14,15). All the neurotoxins produced by *Clostridium botulinum* are immunologically distinct, which suggests significant differences in the amino acid sequences of these toxins. For example, analysis of the partial amino acid sequences of the A and B types has revealed greater homologies between the primary and secondary structure for the light chains than for the heavy chains. The degree of primary structure homology is only 20% for the light chains and 40% for the heavy chains (16). Although they are similar in secondary and tertiary structure, it is believed that differences in the conformation of the neurotoxins at or near the active site may be responsible for the differences in neurotoxicity (17).

Botulinum toxin exists normally in a complex with another protein, hemagglutinin. This accessory protein probably contributes to both conformational stability and resistance to degradation by enzymes. The latter has been shown to be particularly important to the oral toxicity (18) of BTX. While studies have not been reported to support the importance of hemagglutinin to the tissue-reactivity of BTX, it is conceivable that the efficacy of various preparations of the toxin may depend on the stability conferred by this complex formation.

The toxin's effect on neuronal function is apparently limited to the nerve terminal. The toxin, in particular the heavy chain, binds to its membrane receptor, which is apparently localized only at nerve terminals (19). Studies that have examined the binding of radiolabeled toxin molecules suggest that the different serotypes, in particular the type A and B toxins, may bind to different receptor molecules (15,20).

Electrophysiologic studies have demonstrated that botulinum toxins affect different steps in the neurotransmitter release process. Botulinum toxin type B affects synchronization of quantal transmitter release, whereas type A toxin does not (21). Similarly, differences exist with regard to the reversibility of inhibition of calcium-dependent release of neurotransmitter. Introduction of calcium into nerve terminals using a calcium ionophore produces the release of transmitter from synaptosomes poisoned by BTX-A more readily than from those poisoned by BTX-B (22). At the neuromuscular junction, aminopyridine was also more effective at reversal of inhibition produced by BTX-A (23). Ashton and Dolly (22) recently demonstrated that microtubule-dissociating drugs were

effective in blocking the inhibitory effects of BTX-B on neurotransmitter release and ineffective against BTX-A. The differences in the toxic and neurophysiologic effects of type A and B toxins are presumably related to the existence of two distinct receptor sites for these species.

The denervation that occurs at the neuromuscular junction is a consequence of irreversible inhibition of normal neurotransmitter release (24,25). The active form of the toxin produces denervation through a three-step process as suggested by Schmitt et al. and Simpson (24,25). The three-step process involves (1) binding of the heavy chain to the membrane receptor molecule; (2) translocation of the toxin into the nerve terminal via receptor-mediated endocytosis; and (3) irreversible inactivation of normal neurotransmitter release, which is thought to be mediated by the presumed enzymatic properties of the light chain of the toxin. Preliminary studies have suggested that the toxin may be a protease, possibly with zinc dependence (26).

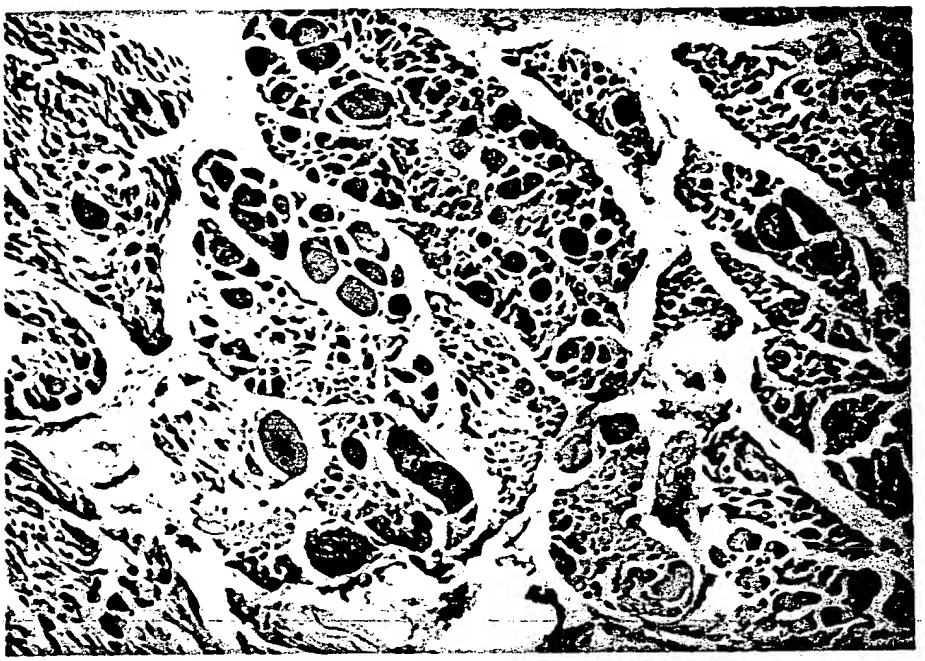
Histological Effects on Muscle Fibers

Within 7 to 10 days of injection, collateral axonal sprouting occurs at the terminal axon or occasionally from the distal node of Ranvier (27,28). Within 10 to 14 days, the muscle fibers begin to atrophy. This atrophy continues to develop over a 4 to 6 week period. Figure 1A–C shows the fiber atrophy achieved in an albino rabbit longissimus dorsi muscle 4 weeks after injection of 10 IU of BTX compared with a saline-injected control specimen. Note that there is not only fiber atrophy as indicated by generalized reduction in fiber diameters but also a large degree of *fiber size variability*. Collateral axonal sprouts reestablish proximity to neuromuscular junctions within a period of several months after injection (29). This sprouting phenomenon is of interest and may provide future insight into the changes in physiologic responses after repeated injections into muscle (see below).

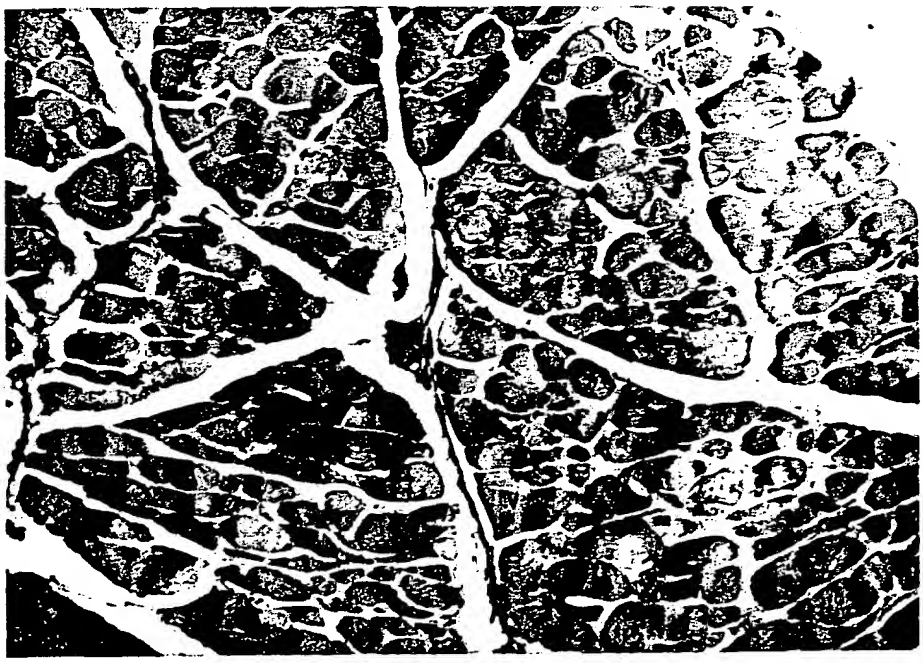
During a period of 3 to 4 weeks after injection, there is considerable spread of acetylcholinesterase (AChE) staining activity on the sarcolemma of muscle fibers. This diffuse AChE staining persists until the 12th to 16th week postinjection, after which there is considerable reduction of staining. After 5 months, AChE activity again becomes confined to the neuromuscular junction, the only area the stain reacts with in noninjected muscles. Spread of AChE staining activity is also correlated with spread of AChE receptors on sarcolemma.

Acetylcholine Nicotinic Receptor Density and Distribution in Response to Denervation

Innervation is known to strongly influence protein synthesis in striated muscle and, in particular, the density and distribution of nicotinic acetylcholine receptors (nAChRs) and AChE (30). The nicotinic receptor is the element responsible for transducing the signal carried by acetylcholine (ACh) into end plate potentials and ultimately muscle contraction. Acetylcholinesterase on the other hand is localized at the end plate at high concentrations and serves to rapidly hydrolyze and inactivate ACh that is released into the synaptic space. Dramatic influences on the density and distribution of these critical elements of the neuromuscular junction are observed during development and synaptogenesis and following denervation. Normally, nAChRs are localized, almost exclusively, to the neuromuscular junction (NMJ) end plate regions of striated muscle (31). In mature striated muscle either before synapse formation or following denervation, nAChRs are observed over the entire muscle surface (32,33). Miledi (34) observed that 10 weeks after complete denervation of frog sartorius muscle, marked hypersensitivity to ACh was detectable over

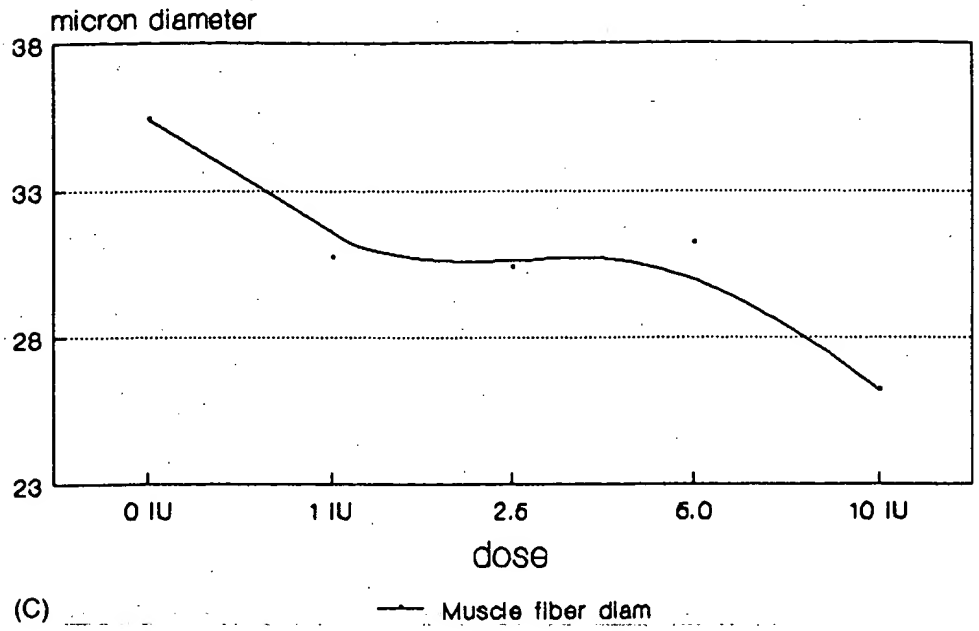


(A)



(B)

Figure 1 Dose-dependent muscle fiber responses: muscle fiber atrophy at the injection site (original magnification 4×). (A) Fiber atrophy is seen after botulinum toxin injections is longissimus dorsi of albino rabbit. (B) Saline control. (C) With increasing doses of botulinum toxin, the degree of fiber atrophy at the injection site increases. (Average fiber diameter represented $n = 3200$ for each point.)



(C)

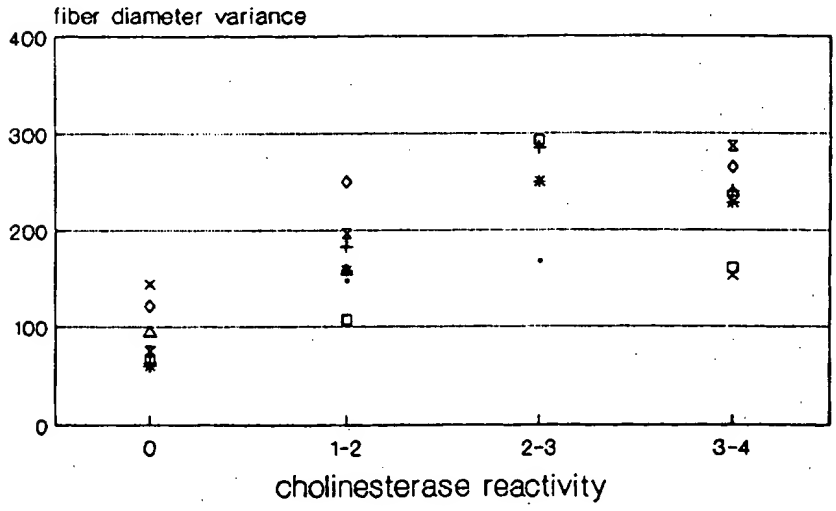
the entire muscle surface. This hypersensitivity was still observed after 158 days, despite the marked muscle atrophy evident at this time. Hypersensitivity to ACh was also observed after partial denervation, a condition more germane to BTX treatment. Consistent with these early observations, Goldman and Stape (35) have reported that denervation of rat soleus muscle resulted in unequal distribution of nAChR subunit RNAs in extrajunctional areas of the muscle as compared with normally innervated muscle, where the RNAs were localized below the end plate region of the muscle membrane. These observations are consistent with the fact that normal innervation suppresses extrajunctional nAChR gene expression and that denervation removes this trophic influence. These observations are in agreement with the spread of AChE staining seen following partial denervation with BTX.

Normal reversal of the effects of BTX is associated with nerve terminal sprouting and reestablishment of myoneuronal junctions. The trophic effects of regeneration of these junctions has been studied in animal models (36–38) and human tissue (39).

Muscle Fiber Morphometric Studies and Histochemistry after Injection

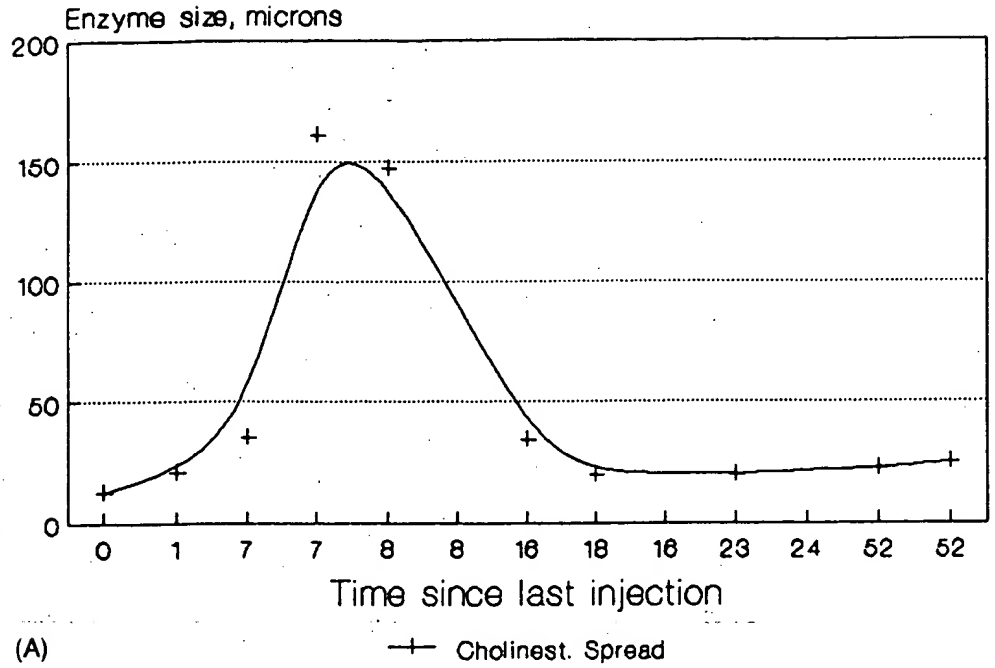
The fiber atrophy measured as reduced fiber diameter, and diameter variability on cross-sectional analysis, is a reversible phenomenon with recovery over a 4- to 6-month period. Spread of AChE on human muscle fibers 5 weeks after BTX injection to the orbicularis oculi muscle is seen in Figure 2A. Figure 2B demonstrates the correlation between fiber size (diameter) variability and cholinesterase staining pattern 5 weeks after injection in albino rabbit muscle. Clinically, this correlation is helpful in assessing therapeutic BTX effects in muscle biopsies. These findings are consistent with those found in human muscle after BTX injections (39–41). Seventeen orbicularis oculi muscle specimens taken from patients during ptosis and myectomy surgery were evaluated for cholinesterase staining characteristics and fiber variability (39). Diffuse cholinesterase activity started at weeks 3 to 4, and this staining pattern was maintained through 3 to 4 months after injection (Fig. 3A). In all patients studied after 6 months, cholinesterase staining was confined to the NMJs and could not be distinguished from that seen in controls. It is also

(A)



(B)

Figure 2 (A) Acetylcholinesterase staining in human Orbicularis oculi muscle. Spread of acetylcholinesterase is seen on human muscle fibers 5 weeks after injection of botulinum toxin. A similar effect was previously described in animal muscle fibers (see Fig. 6). (B) A direct correlation between fiber size variability and acetylcholinesterase spread characteristics on muscle biopsies evaluated from the animal study.



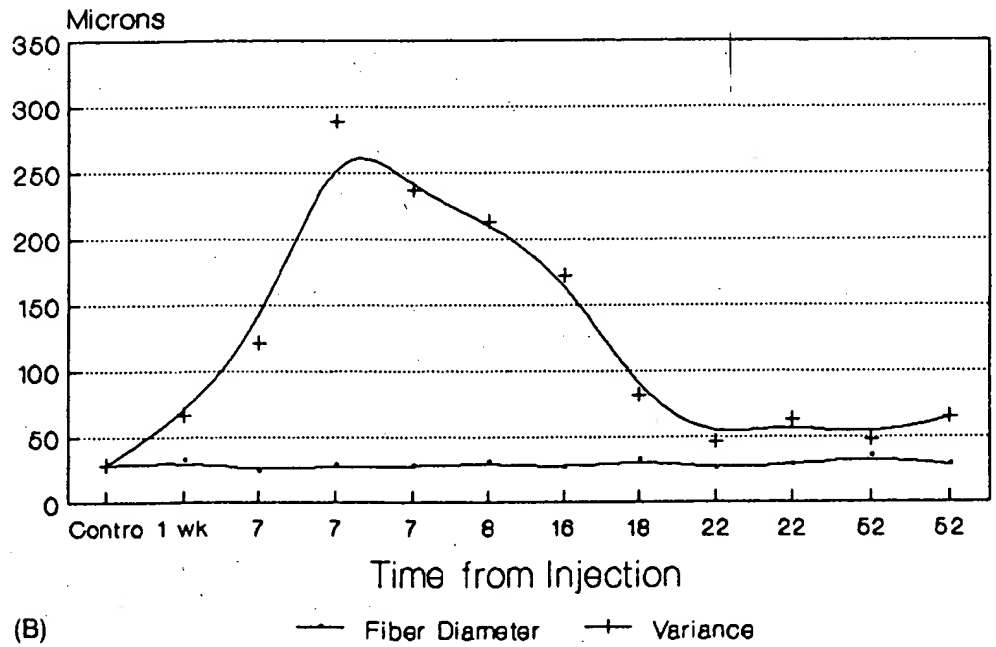


Figure 3 Cyclic histological changes after botulinum toxin (BTX) injection in human muscle (39). (A) Denervation reflected by cholinesterase spread. (B) Variations in orbicularis oculi muscle fiber size after BTX injection. The duration of action of BTX appears to correlate with fiber variability and chlolinesterase spread characteristics seen in human orbicularis muscle specimens. The results of these specimens were evaluated with respect to the last injection of BTX. The specimens were obtained at the time of ptosis surgery and myectomy surgery in patients being treated by this technique for their diseases. The fiber morphometric response and cholinesterase activity correlate with the clinical duration of action. The time scale on the graphs is in weeks.

of note that fiber size variability appeared to correlate temporally with cholinesterase spread pattern. Fiber size variability appeared to be transient, lasting 3 to 12 weeks after injection (Fig. 3B). Temporal relationships between these histologic changes correlate with the duration of action achieved with therapeutic doses of BTX, which varies between 10 and 16 weeks in most dystonia applications (42).

Another important goal in evaluating human muscle specimens after the injection of BTX is assessing long-term muscle fiber effects. If human orbicularis oculi muscle specimens were not injected within 6 months of biopsy, there was no difference in fiber size or cholinesterase staining pattern compared with the control specimens taken from routine ptosis surgery (39). Chronic denervation and muscle fiber atrophy does not appear to occur with repetitive use of the toxin. However, there appear to be permanent changes within the myoneural junctions, with increased number of preterminal axon sprouts and multiple projections into the myoneural junctions, as will be demonstrated later in this chapter (see below, "Motor Nerve Terminal Morphology Following Botulinum Toxin Type A Injection"). Although changes may be present at the neuromuscular junction with respect to sprouting, there was no substantial residual atrophy after multiple injections of BTX. Such data appear to indicate that the reinnervation process after BTX administration is nearly complete, and that permanent trophic changes within muscle fibers do not occur.

Adenosinetriphosphatase (ATPase) enzyme histochemistry on animal muscle tissues injected with BTX has demonstrated slight alteration in the pattern of muscle fiber types. The number of type I muscle fibers slightly increased after the injection. However, the most significant finding was type grouping (see Figure 4). Fiber size variability was seen

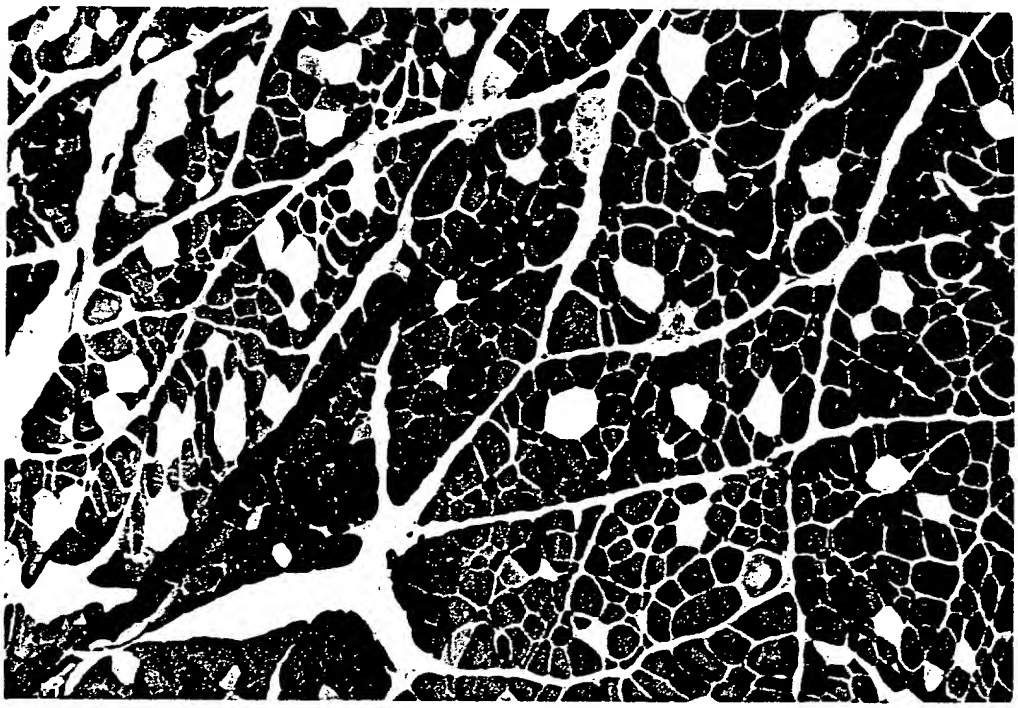


Figure 4 Adenosine triphosphatase stain at pH 9.4. The amount of type I fiber grouping appears to be greater after injection of botulinum toxin in comparison with controls. However, the total number of type I and type II fibers did not seem to be substantially different from that seen in controls. This appearance is consistent with denervation.

in both type 1 and type 2 muscle fibers. Muscle fibers that belong to the same motor unit are morphologically, histochemically, and physiologically similar. Type 1 fibers are innervated by low-threshold motor neurons, while type 2 fibers are innervated by higher-threshold neurons. They can be differentially identified using ATPase enzyme histochemistry. In the normal condition, there is a mosaic-like pattern to the distribution of innervation by each motor unit. Although this pattern varies between different muscle groups, it can be somewhat likened to a checkerboard. The successful reinnervation of denervated muscle from nearby collateral axon sprouts by a different motor unit type will convert the reinnervated muscle fiber to that type. As a consequence, the distribution of muscle fiber types changes. Fiber type grouping is a common occurrence. The altered mosaic pattern of fiber typing in animal muscles injected with BTX, as observed using ATPase enzyme histochemistry, is consistent with that observed in denervation. These changes occur more slowly and are not as dramatic as the spread of AChE activity seen in animal tissues 3 to 5 weeks after BTX injection.

The pattern of oxidative enzyme activity with nicotinamide adenine diaphorase histochemistry was also changed in animal muscle treated with BTX. The muscle fibers appeared "moth-eaten," with a focal, irregular loss of enzyme activity. This finding has been associated with an abnormal redistribution of mitochondria within the affected muscle fibers. Neither target nor targetoid fibers were observed. As with the alterations of fiber typing, the dysmorphic changes in oxidative enzyme activity are compatible with a denervative process.

MOTOR NERVE TERMINAL MORPHOLOGY FOLLOWING BOTULINUM TOXIN TYPE A INJECTION

Botulinum toxin blocks neuromuscular transmission and produces functional muscle denervation (27,43,44). The motor axon remains in anatomical contact with the muscle end plate, but because neuromuscular transmission is blocked, the muscle is paralyzed. In rats and other experimental mammals, two morphological changes develop at the NMJs following functional denervation by BTX. In proximal muscles, there is conspicuous motor axon sprouting. In more distal muscles, expansion of the end plate region is more pronounced, through both responses can be seen in any muscle.

Sprouts develop from multiple sites of the preterminal axon (Fig. 5), including the terminal axonal arborization over the end plate region (ultraterminal sprouts), the axon immediately proximal to the end plate (terminal sprouts), and the nodes of Ranvier of preterminal axons (preterminal sprouts). By electron microscopy, the axon sprout follows the inner layer of a basal lamina, either of muscle or of the enveloping Schwann cell (K. Alderson, unpublished data). The sprouts are otherwise nondirected, growing through the muscle, but not generally terminating at muscle end plates (dead ends). There has been mention in the literature of these sprouts terminating at end plates (44). The sprouts decrease in abundance after several months, though the process of sprout loss is not well understood. Expansion of the end plate is more apparent in distal muscles following BTX exposure, with increased branching of the terminal axonal arborization over the end plate and enlargement of the cholinesterase-containing end plate region, from the normal length of 30–60 μm to more than 120 μm (Fig. 6 and Fig. 2A). The end plate region is not always continuous, with cholinesterase-containing regions separated by 5–15 μm. Synaptic vesical antigenicity is present along the extended branches of the axonal arborization (Fig. 7), suggesting that the expanded neuromuscular junction is capable of neuromuscular transmission.

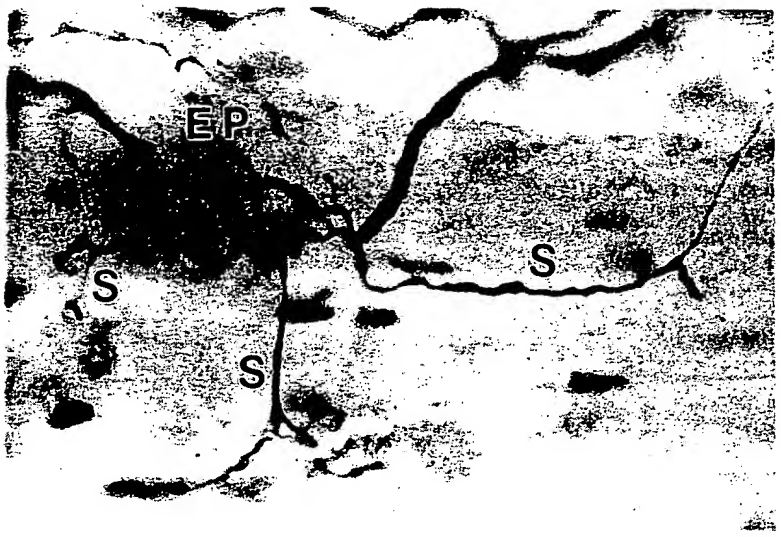


Figure 5 Axonal sprouts in the rat rhomboid muscle 14 days following injection of botulinum toxin type A. Exuberant ultraterminal axonal sprouts (S) arise from the terminal axonal arborization over the end plate (EP) region (silver-cholinesterase, × 500).

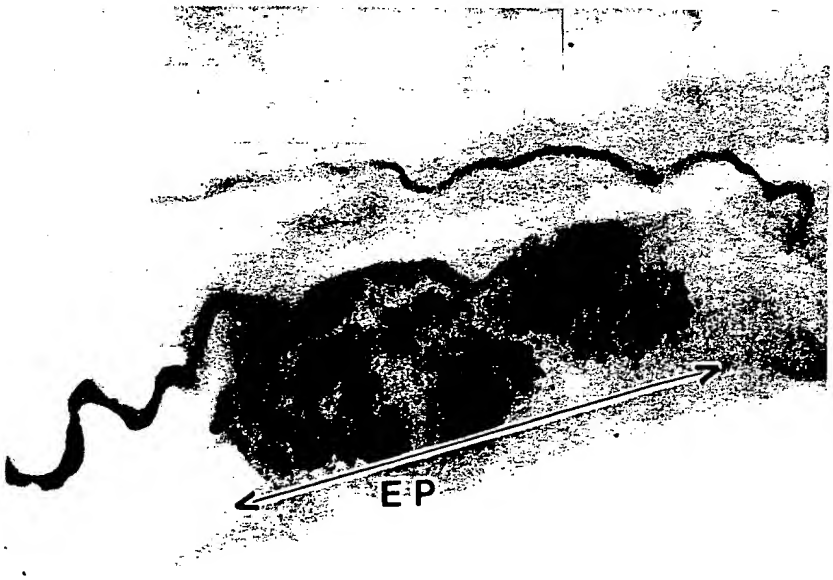


Figure 6 Expansion of the end plate (EP) in the rat soleus muscle 14 days following botulinum toxin injection into the muscle. The diameter of the cholinesterase-containing region has increased from the normal 30–60 μm to, here, 120 μm (arrows). Synaptic vesicle antigenicity is the fuzzy area surrounding the axonal processes over the terminal arborization (SCI, using an antibody to the synaptic vesicle protein synaptophysin). (× 600.)

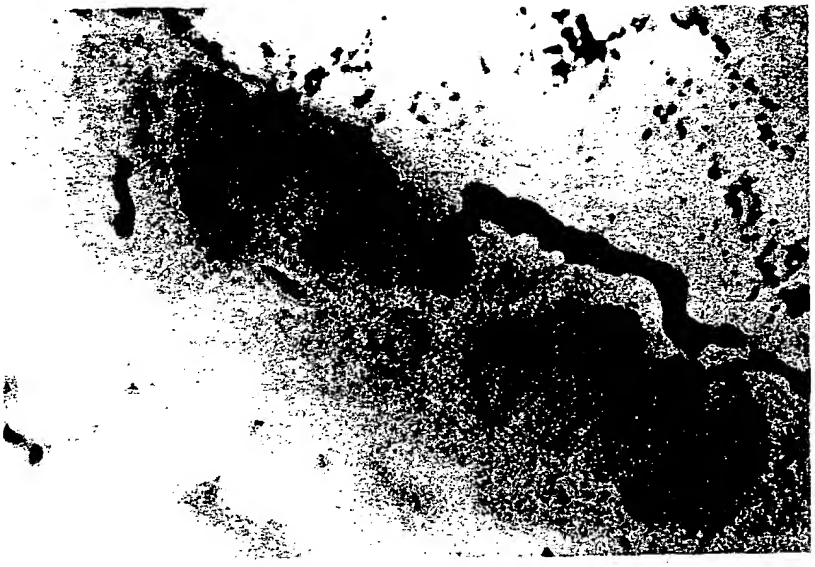


Figure 7 Two cholinesterase-containing end plate regions are separated by a non-cholinesterase-containing muscle membrane. An axonal process extends to the second end plate region. Synaptic vesicle antigenicity surrounds the axonal processes over the terminal arborization (SCI, using an antibody to the synaptic vesicle protein synaptophysin, × 800).

Human Terminal Motor Axon Configuration Following Botulinum Toxin Injection

Some patients with disabling blepharospasm in whom BTX injections or medical therapy is not successful in controlling the spasms elect orbicularis oculi myectomy as treatment (45). Surgical specimens of this muscle were evaluated to study the effect of BTX on terminal motor axons in humans (29,46).

Orbicularis oculi muscle from three groups of patients, age 35–81 years, was evaluated:

1. Nine patients with blepharospasm who had previously received 2 to 19 botulinum toxin type A (BTX-A) injections 5 weeks to 3 years before undergoing orbicularis oculi myectomy as treatment for blepharospasm. Four of the 9 patients failed to achieve sustained responses to repetitive BTX treatment and required an increased dose on subsequent injections. One patient had severe and prolonged paralysis following his second injection, and refused further injections. The other four had continuing response to injections.
2. Two patients with blepharospasm without prior BTX therapy electing orbicularis oculi myectomy.
3. Six "normal" patients without blepharospasm having cosmetic blepharoplasty.

Normal Human Orbicularis Oculi Terminal Motor Axons

Terminal motor axon and NMJ morphology in muscle not exposed to BTX, in both "normals" and patients with blepharospasm, was identical (Fig. 8) (29,46). Single preterminal axons exit the intramuscular nerve. All axons are myelinated up to the muscle end plate region. There are no unmyelinated axons. Most preterminal axons are unbranched and innervate a single muscle fiber; fewer than 10% of preterminal axons branch

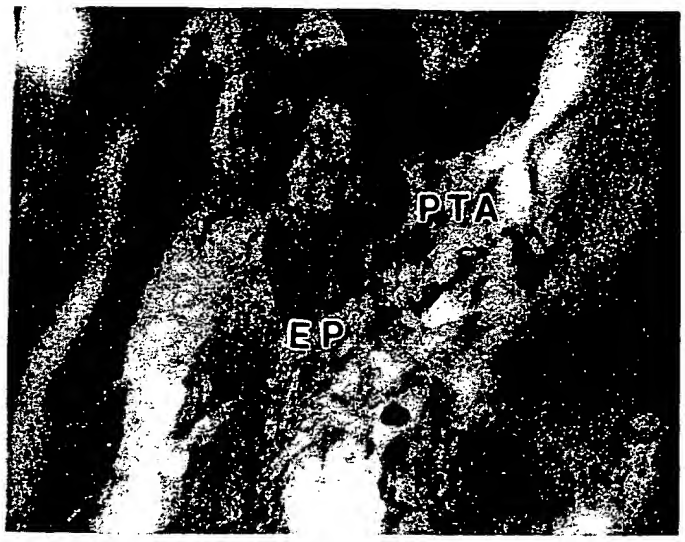


Figure 8 Normal orbicularis oculi muscle terminal motor axons. The preterminal axon (PTA) is myelinated as it leaves the intramuscular nerve, and myelination extends to the muscle end plate (EP) region (× 250, SCI using an antibody to PO).

to innervate more than one muscle fiber. Muscle end plates are not segmented, with a diameter between 6 and 34 μm, mean 20 μm (29). Each muscle fiber contained a single end plate region.

Motor Axons and Neuromuscular Junctions Following Botulinum Toxin Exposure: Sprouts

As expected on the basis of animal data, axonal sprouts develop following BTX injections into human orbicularis oculi (Fig. 9) (46). Preterminal, terminal, and ultraterminal sprouts are present in all BTX-treated muscle, though of greater incidence in muscles having more injections. The sprouts are almost uniformly unmyelinated. Some sprouts clearly end in a small bulbous dilatation, presumed to be the growth cone (Fig. 10).

Alteration of End Plate Size and Arrangement

As in animal muscle, cholinesterase-containing end plate regions expand in the orbicularis oculi following BTX injections (Fig. 2) (29). Segmented end plates form, with cholinesterase-containing regions separated by relatively long areas of non-cholinesterase-containing muscle fiber membrane. Each region of the segmented end plate is innervated by axon processes from the terminal or ultraterminal region of a single axon (Fig. 11). It appears that the axonal sprout can induce the formation of an end plate region on the underlying muscle membrane.

Very small end plates are also present, innervated by thin, unmyelinated axonal processes. End plate diameter ranged from 3 to 65 μm. Though the mean end plate diameter in BTX-treated muscle is about 20 μm, similar to untreated muscle, the preponderance of larger and smaller end plates contributes to a significantly larger standard deviation. The number of end plates in an area of muscle increases, and the number of end plate regions identified on an individual muscle fiber increases, from one in normal muscle to five or more in BTX-treated muscle.

Figure 9 Unmyelinated axonal sprouts (S) extend from preterminal axons into the orbicularis oculi muscle, in a muscle receiving 14 botulinum toxin injections (× 150, SCI using an antibody to PO).

Figure 10 Some sprouts (S) from preterminal axons (PTA) end in small dilations, presumed to be the growth cones of the axon, in a muscle receiving nine injections (× 250, SCI using an antibody to PO).

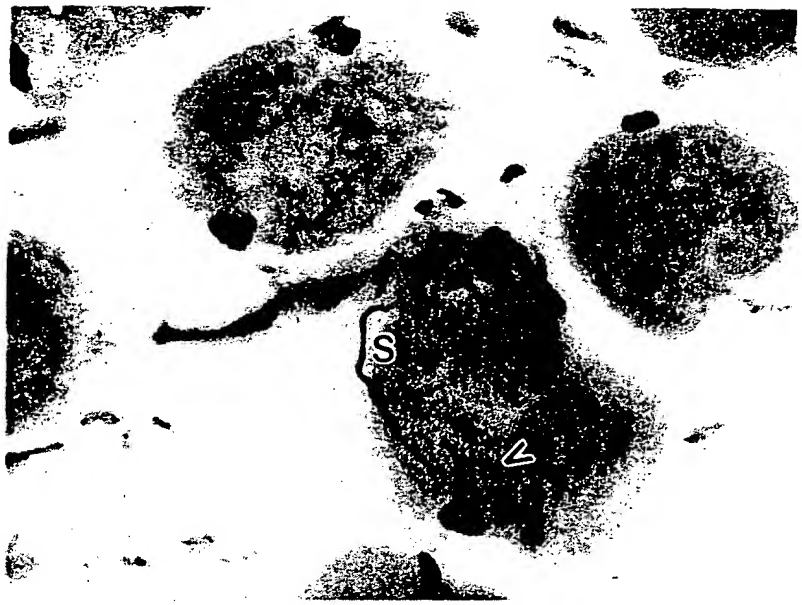


Figure 11 Segmented end plates. An unmyelinated ultraterminal process (S) extends to a separate end plate region (arrowhead) (× 600, SCI using an antibody to PO).

Axonal Collaterals

Axonal collaterals develop in disorders that cause partial anatomical denervation (29). With loss of some but not all axons to a muscle, the remaining motor axons "sprout," grow toward, and reinnervate denervated muscle fibers (47,48). This normal repair process increases the number of muscle fibers innervated by a single motor axon. Unexpectedly, axonal collaterals develop abundantly in BTX-treated muscles (Fig. 12). Axonal collaterals are generally thin and unmyelinated. Collaterals extend to end plates of all sizes. Axonal collaterals are present in muscle from all patients receiving botulinum toxin, whether they had few or many injections. Axonal collaterals are present as early as 5 weeks following the second injection and persisted in patients who had their last injection 3 years previously.

Dual Axonal Innervation of Individual Muscle Fibers

Some muscle fibers in BTX-treated muscle contain more than one end plate, each innervated by separate preterminal motor axons (Fig. 13) (29). Dual innervation of muscle fibers has not been identified previously in adult mammals following functional denervation in experimental animals or in human neuromuscular disease. Dual innervation of muscle fibers by separate preterminal axons on separate end plate regions may develop because functional denervation makes the muscle "receptive" to responding (in some unknown manner) to an axonal process to form an end plate region. The axonal process can arise from the original innervating axon, producing segmented end plates, or from a separate preterminal axon, producing dual innervation. The ability of functionally denervated muscle to allow formation of a second end plate has been demonstrated experimentally following BTX administration and subsequent implantation of other motor axons at a distance from the denervated end plate (49). Such experimental evidence supports the

hypothesis that repeated functional denervation in humans allows axonal sprouts to induce formation of new end plates on the functionally denervated muscle fiber, thus forming collateral innervation.

Axonal collaterals form readily in partial denervation associated with loss of motor axons (48–53). The reduction in the number of motor axons is readily appreciable as decreased density of axons in the intramuscular nerve in experimental partial denervation and in human denervating disease, such as amyotrophic lateral sclerosis or peripheral neuropathy. It is unlikely that there is a significant loss of motor axons following BTX injection, as the morphology and density of axons in the intramuscular nerve is normal, whereas a reduction would be expected if collateral sprouting developed because of a partial denervation from loss of axons.

Small end plates innervated by collaterals could represent either end plates on atrophic fibers or end plates formed under the influence of BTX. The presence of multiple end plates on a single muscle fiber, as well as segmented end plates, suggests that sprouts can induce the formation of new end plates on muscles "functionally denervated" by BTX. Two end plates on one muscle fiber, each innervated by different axons, can be induced and maintained experimentally in mammals by implanting a motor axon 5 mm from an end plate functionally denervated by BTX (47). The arrangement of the orbicularis oculi, with widely scattered regions of end plates and sprouts developing from motor axons in each region, may facilitate the development and persistence of multiple end plates, so that a single muscle fiber can be innervated at separate sites by collaterals from more than one axon.

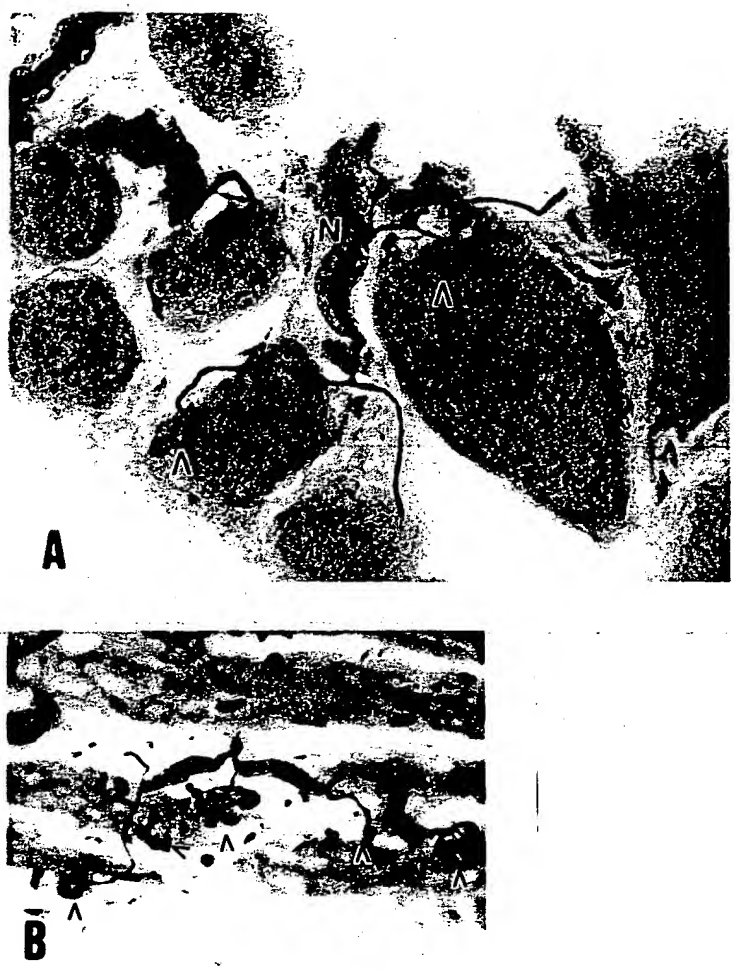
Although evaluation of sprouting is subjective, ranking of patients by the relative abundance of unmyelinated axons and noncollateral sprouts is justified, as sprouting clearly varied among patients. Since the relative abundance of sprouts increases with the total number of injections, the effect of botulinum injection on sprouting from terminal motor axons may be cumulative.

Long-Term Muscle Fiber Changes Following Repetitive Botulinum Toxin Injections

Muscle fiber atrophy, as well as gross atrophy of the muscle, develops following BTX administration in both experimental animals and humans (39,54,55). The atrophy reverses readily in experimental animals. After several months, the histological appearance of the muscle returns to "normal." To determine if muscle fiber atrophy persists in humans, the histological appearance of the same muscles described above was studied and compared with normal orbicularis oculi muscle and muscle from patients with blepharospasm not previously treated with BTX injection. Mean fiber diameters and variance were not different from controls if specimens were taken from patients who had not been injected within 4 months of the biopsy (39,54). There are no long-term, persistent changes in orbicularis muscle fiber appearance following BTX injections, and BTX-induced muscle fiber atrophy in humans is reversible (Fig. 14).

The Maintenance of Altered Neuromuscular Interactions Following Botulinum Toxin Injection

The normal NMJ is not static but is constantly remodeled. Axonal sprouts spontaneously grow from the terminal axonal arborization, and other axonal processes retract. Overall, the area of neuromuscular contact remains relatively constant. In muscles treated with



BTX, in contrast, the potential sites of neuromuscular contact seem to increase. There are more end plates on muscle fibers, and many of the end plates are larger than normal. Why these synapses are not remodeled is not known. Investigation of the persistence of these synapses may have implications for understanding abnormal synapse maintenance in disease.

In summary, BTX injection into the human orbicularis oculi muscle produces axonal sprouts, variation in the size of end plates, with expansion of some end plates and the apparent formation of new and smaller end plates, and reorganization of muscular innervation. Repeated and long-standing functional denervation allows axonal sprouts to induce the formation of nascent end plates on the functionally denervated muscle fibers, thus producing axonal collaterals. The number of muscle fibers innervated by a single preterminal axon increases. A single muscle fiber may be innervated by more than one preterminal axon (Table 1). Muscle fibers do not remain atrophic despite more persisting changes in the motor axon terminals.

Morphological abnormalities of terminal motor axons and NMJs increase with the number of injections or the severity of the paralysis, and seem to be persistent. These observations may have implications for understanding the stability of the NMJ and may have clinical relevance as well.

Figure 12 Axonal collaterals. (A) Terminal innervation = at least 2. Unmyelinated collaterals originate at nodes of Ranvier (N) of a preterminal axon and from the terminal axon, and extend to end plates on adjacent muscle fibers in a patient receiving nine injections (× 500, SCI using an antibody to PO). (B) Terminal innervation = 5 in a patient receiving 2 injections. Unmyelinated and myelinated collaterals (arrowheads) extend from nodes and from the terminal axon (× 200, SCI using an antibody to PO). (C) Terminal innervation = 7 in a patient with 18 injections. (× 200, silver-cholinesterase.)

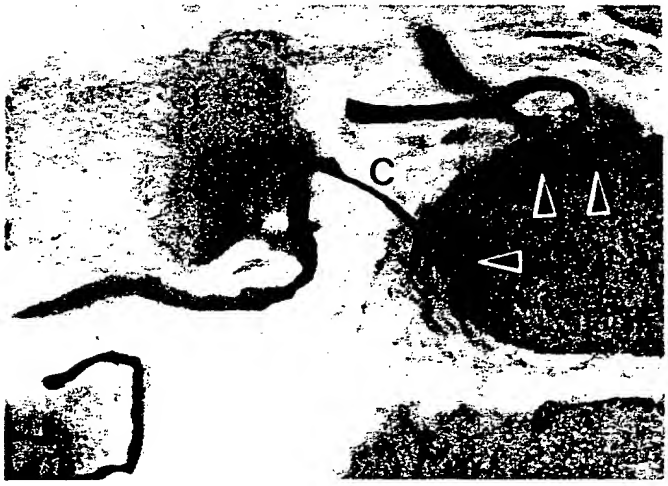


Figure 13 Dual innervation of muscle fibers. An ultraterminal collateral (C) extends to an end plate on a muscle fiber also innervated by another preterminal axon (× 600, silver-cholinesterase).

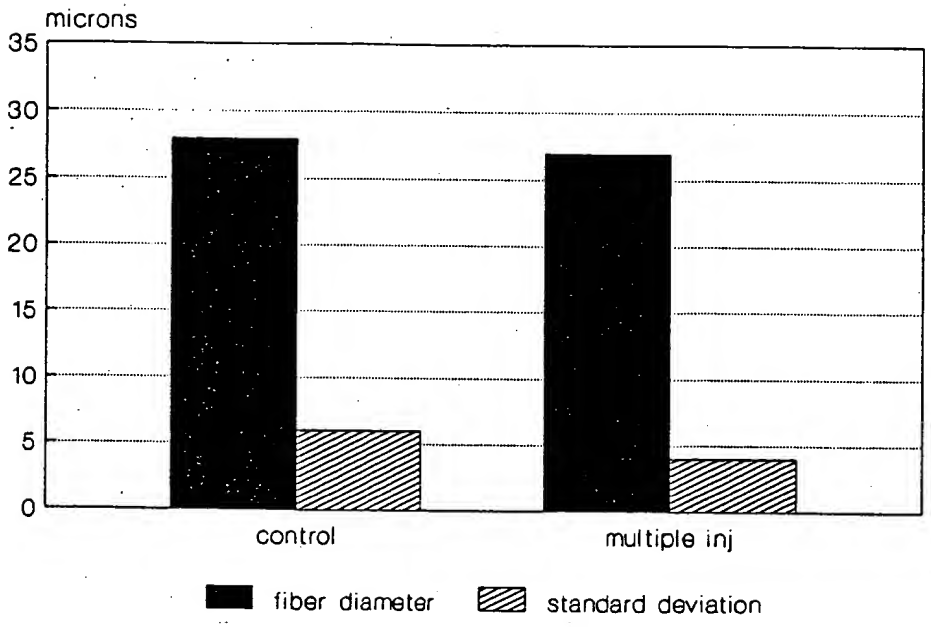


Figure 14 Muscle fiber size and variance after repeated injections. Long-term muscle fiber atrophy does not occur after repeated injections but does occur within 3–12 weeks and is reversible.

DIFFUSION OF BIOLOGICAL ACTIVITY FROM THERAPEUTIC INJECTION SITES: CLINICAL SIGNIFICANCE

Strabismus

"Strabismus" is an ophthalmic term that describes pathological misalignment of the eye. Therapy with BTX had been advocated by Scott (1–3) as an alternative to conventional extrocular muscle surgery because of the greater simplicity of the procedure. Unfortunately, therapy offered by the toxin injection is temporary, and the injection needs to be repeated in a majority of cases to maintain ocular alignment. The ability of the clinician to target the desired muscle exactly is limited by BTX diffusion away from the site of injection. Treatment usually involves injection of the medial rectus or lateral rectus muscles (horizontal rectus muscles) to treat horizontal deviations of the eyes. Diffusion of BTX into the vertical rectus muscles resulting in complications is common (1–3). Induced vertical deviation of the eye when horizontal deviations are being treated is clearly a limiting factor, occurring in approximately 15% of cases (3). Ptosis from intraorbital

Table 1 Summary: Morphology of Botulinum Toxin-Induced Sprouts In Humans

"Nondirected" sprouts following muscle or Schwann cell basal lamina
Expansion of muscle end plate regions
Increased number of end plates on a single muscle fiber
Increased number of end plates in regions of muscle
Axonal collaterals
Increased number of muscle fibers innervated by a single motor axon
More than one axon innervating a single muscle fiber with no loss of motor axons and normal muscle histochemistry

injections of BTX into extraocular muscles with diffusion into the levator palpebrae superioris muscle is also very common (see below).

Blepharospasm and Meige Syndrome

Over the past 12 years, BTX therapy has expanded to treatment of focal and segmental dystonias (4–11). In these conditions, neurologic imaging and even postmortem examination usually do not reveal structural lesions within the brain.

The first dystonia for which BTX therapeutic technology was used was benign essential blepharospasm and Meige syndrome (4). This form of neurologic blepharospasm is associated with blinding involuntary eyelid closure that leads to debilitation and desperation. In the past, therapy had included the use of neuroleptic medications, which were usually ineffective (56). Surgical therapy involving transection of the facial nerve or removal of the orbicularis oculi muscle in surgical stripping procedures (57,58) was tried, but in many patients this approach is only partially effective, and it is occasionally associated with undesirable complications.

Botulinum toxin has become the only consistent therapy for neurologic forms of blepharospasm. The eyelids are injected with small quantities of BTX (15–75 IU), producing an effective weakening of the protagonist muscle of eyelid closure, the orbicularis oculi. This muscle must be injected every 3 months in the case of essential blepharospasm and Meige syndrome and every 5 months in blepharospasm associated with hemifacial spasm and aberrant regeneration of the seventh cranial nerve (60).

As BTX therapy for this disease has proven to be effective (4,5,7), it has also been limited by several complications, including ptosis, exposure keratopathy, diplopia (double vision), and epiphora (tearing). Ptosis is defined as a drooping of the upper eyelid causing encroachment of the upper lid on the visual axis and effectively decreasing vision. This complication is generally transient and disappears as the denervative effect of the botulinum toxin wears off. It occurs in approximately 10% of patients treated and is particularly prone to occur when the injections of BTX are made close to the superior sulcus. Although patients already have eyelid disease causing obstruction of vision, visual function is further impaired by the ptosis. Understanding the cause of this complication is important to the effective application of the therapy. The occurrence of ptosis is thought to relate to diffusion of the toxin from the injected orbicularis oculi muscle into the superior orbit, effectively weakening the retractor of the upper eyelid, the levator palpebrae superioris muscle (5). Weakening of the muscular portion of the levator causes dropping of the upper lid margin. Given that the control of upper eyelid movement is primarily a balance between the actions of the orbicularis muscle and those of the levator palpebrae superioris muscle, effective application of BTX for blepharospasm involves confining the biological effect of the toxin to the orbicularis muscle, the protagonist muscle causing the abnormal movement. *The anatomical configuration of the muscles of the upper eyelid allows selective weakening of the orbicularis muscle and, therefore, effective therapy with BTX. The long tendon of the upper eyelid retractor, the levator aponeurosis, extends along the undersurface of the orbicularis muscle into the tarsal plate. In that the muscular portions of the levator palpebrae superioris muscle are remote from its antagonist (the orbicularis muscle), there is less likelihood that the BTX will diffuse into the elevator of the eyelid. This anatomical distance between eyelid orbicularis (pretarsal orbicularis) and levator muscle provides an anatomic explanation for therapeutic success in selectively targeting the orbicularis muscle in treating blepharospasm. This explanation also provides insight into the cause of this complication and a method to guard against its occurrence.*

Another complication associated with therapeutic injections of BTX into the eyelids is diplopia. This occurs less commonly (< 5% of patients) and is transient, lasting several days to several weeks. Nelson and her co-workers (61) have linked this complication to injections in the lower lid, particularly the inner aspect of the lower lid. The reason for the occurrence of diplopia appears to relate to the anatomical proximity of the inferior oblique muscle to the inner portion of the lower lid. The inferior oblique muscle arises in the very anterior portions of the medial orbit and penetrates the major fascia of the lower lid (capsulopalpebral fascia) very close to the cutaneous surface of the medial lower lid. Injection into the inner aspects of the lower lid brings the toxin within several millimeters of this muscle, so that the toxin can readily diffuse into this region and produce this complication. Avoiding medial lower lid injections reduces the incidence of diplopia (61).

With respect to the issue of efficacy in the treatment of blepharospasm, it appears that diffusion may again play a role. The location of the injection sites used today was empirically derived and was based on apparent efficacy as well as a strategy that limited complications. The need for multiple injection points adds to the discomfort of the application procedure. Initially, therefore, the effects of injecting only electrically determined motor points of the orbicularis oculi muscle were studied (62). The motor points of the orbicularis oculi muscle are essentially in two locations, the outer portion of the upper eyelid and the medial portion of the lower eyelid. The motor point is defined as the area within muscle that has the lowest threshold for contraction in response to external electrical stimulation. Generally, motor points correspond to points of major motor nerve branch penetration into the muscle proper (62). Comparisons of single motor-point injection with multiple-point injections were made in a series of 10 patients by using the multiple-point injection strategy on one eye and the motor-point injection strategy on the other, each eye receiving the same dose. Eight of the 10 patients found that the eye treated by multiple-point injection technique had a substantially better result than the eye treated with single motor-point injection. The other two patients noted no differences between eyes (62). The results of this clinical study suggested several points:

1. Dose-independent variables are involved in determining efficacy in BTX treatment.
2. Spreading the toxin throughout the muscle may have increased toxin diffusion throughout most of its innervation zone.
3. The results tend to negate the opinion that motor points could provide a superior injection location.

Histologic Innervation Zone Analysis of Human Orbicularis Oculi

In an effort to explore the innervation zone, that is, the topographical distribution of NMJs within a muscle, strips of orbicularis oculi muscle taken from the pretarsal portions of the muscle during routine ptosis surgery were analyzed for concentration and distribution of the NMJs using an AChE enzyme histochemistry method (62). Although only the pretarsal portions of the muscle were analyzed in this study, this portion of the muscle is exactly over the lateral motor point of the upper lid. The results indicated that the NMJs were diffusely distributed through the entire upper portion of the orbicularis muscle (Fig. 15). This finding clearly correlates with the large degree of facial nerve projection and ramification into the orbicularis muscle from temporal, zygomatic, and buccal branches. This observation leads to the hypothesis that multiple injection points were preferable because this injection technique tended to cover more of the innervation zone of the orbicularis muscle as compared with a single-point large-dose injection. For orbicularis muscle, this appears to be a plausible explanation for the superiority of the multiple-point

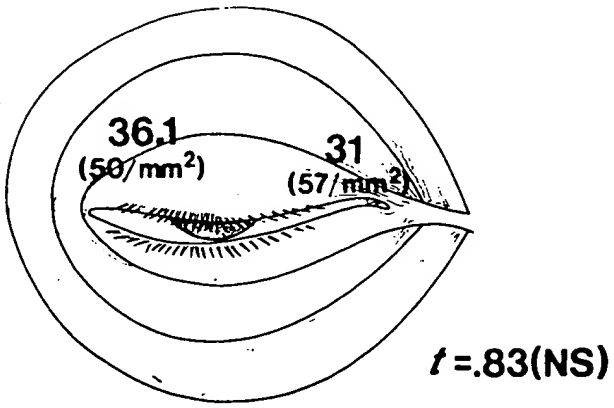


Figure 15 Distribution of the neuromuscular junctions within the upper eyelid orbicularis oculi muscle. Acetylcholinesterase staining can be used to map the distribution of neuromuscular junctions (innervation zone) within a muscle. Neuromuscular junction distribution was determined for the portion of the orbicularis oculi contained in the upper eyelid from specimens taken to debulk the upper eyelid fold during routine ptosis surgery on a patient never injected with botulinum toxin (62). The distribution of the neuromuscular junctions is diffuse within these muscle strips and specifically not concentrated over the electrically determined motor points for this muscle (62). The diffuse distribution of the innervation zone may be the reason that multiple injections into this muscle for the treatment of blepharospasms are superior to single or "motor point" injections. Large numbers refer to absolute NMJ counts, and small numbers relate these counts according to surface area. The motor point of the upper lid is directly over the lateral pretarsal orbicularis muscle.

injection technique. It appears that diffusing the toxin along the muscle proper was preferable in this particular anatomical location. This study also disproved the notion that motor point of the muscle corresponds to the innervation zone (distribution area of motor end plates within a given muscle).

Adult-Onset Spasmodic Torticollis

Adult-onset spasmodic torticollis is a segmental dystonia involving cervical muscles. The condition can occasionally be associated with Meige syndrome or other forms of head and neck movement disorders. A thickened protruding sternocleidomastoid muscle is the most characteristic finding in these patients on physical examination. Torticollis has variable expression, the most common pattern being a distorted posture with head rotated to a side of shoulder elevation (type 1, type 2) (6). Another variation involves tilting of the head toward the side of shoulder elevation (type 3) (6). Another variation includes involuntary backward tilting of the head (retrocollis, type 4a) or flexing of the head toward the chest (antecollis, type 4b) (6). Patients often develop pain early in the course of the disease, which is frequently progressive. The disease is often associated with involuntary jerking movements of varying frequency and amplitude.

Past therapy has included the use of neuroleptic medications, various forms of myectomy and denervating surgery, and occasionally biofeedback (63,64). Unfortunately, previous medical therapies have not been satisfactory. Surgical procedures are often associated with inconsistent results and occasionally with disfiguring scarring and further impairment in posture.

The application of BTX to the treatment of this segmental disorder has been a great contribution to neurological medicine. It has proven to be the most efficacious therapy for torticollis and can be maintained over a period of years (6,12,23).

The major complication associated with the use of BTX for spasmodic torticollis has been dysphagia (40). Dysphagia is defined as difficulty in swallowing that can occasionally lead to the misdirection of food into the upper airway. Such misdirection can occasionally cause complete upper airway obstruction, which is a medical emergency possibly leading to death. Upper airway obstruction has occurred in at least one patient involved in the North American clinical studies (40). This patient was immediately and successfully treated by the Heimleich maneuver. Other complications have included weakness of the cervical muscles.

The diffusion model has proven to be important in finding a solution to the dysphagia problem. In retrospective studies, dysphagia appeared to be linked to the dose of BTX injected into the sternocleidomastoid muscle (Fig. 16): data indicated that doses in excess of 100 IU were associated with the complication (Table 2). In prospective data analysis, limiting the dose to the sternocleidomastoid muscle was shown to markedly decrease the incidence of this complication. The complication rate reported initially in our studies was comparable to those noted in other studies, approximately 15% (8,11,40,42). Limiting the sternocleidomastoid dose to less than 100 IU during an injection session reduced the

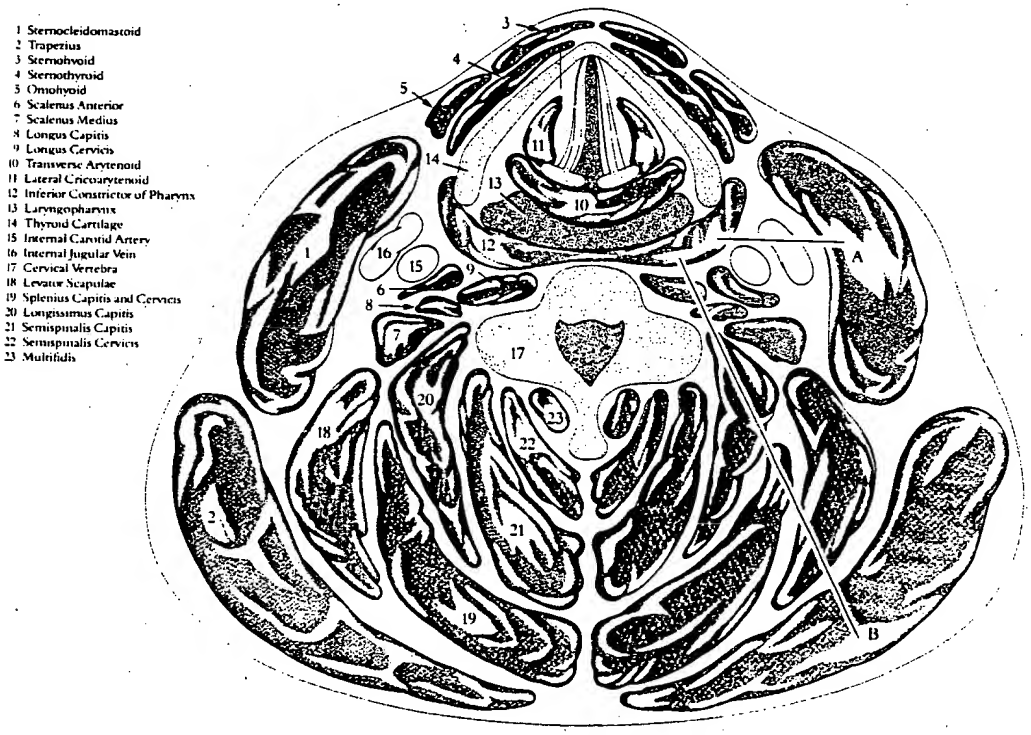


Figure 16 Dysphagia and the treatment of adult-onset spasmodic torticollis. Dysphagia can result from diffusion of botulinum toxin into the peripharyngeal musculature. This complication particularly occurs when high doses of botulinum toxin are given to the sternocleidomastoid muscle, which directly overlays the peripharyngeal muscles. This complication occurs as a result of direct spread of biological effect from injected sternocleidomastoid muscle into the deeper structures of the neck. Table 2 outlines the results of the clinical study linking dysphagia to the dose of botulinum toxin given over the sternocleidomastoid muscle.

Table 2 Comparison of Botulinum Toxin Dose and Injection Strategies in Patients Who Later Experienced Dysphagia and Those Who Did Not

	Dysphagia	No dysphagia
Sternocleidomastoid dose		
No. injection	7	42
Median dose	150	100
Interquartile range	10	150
Wilcoxon test	$Z = 2.22$	$p = 0.026$
Total dose		
No. injection	7	42
Median dose	160	150
Interquartile range	25	100
Wilcoxon test	$Z = 0.75$	$p = 0.45$

incidence of this complication to less than 2%. A plausible explanation for these findings is that diffusion of toxin from the sternocleidomastoid muscle into the peripharyngeal musculature resulted in weakening of the muscles involved in the swallowing reflex. The sternocleidomastoid muscle lies directly over the peripharyngeal musculature, while the other muscles usually injected in the treatment of spasmodic torticollis (levator scapulae, posterior scalene, trapezius, splenius capitis, splenius cervicis, and others) are more posterior to and remote from the pharyngeal muscles.

Since it appears that *dysphagia was secondary to toxin jump*, that is, toxin spread and diffusion from the sternocleidomastoid muscle, this complication can be limited by reducing the dose in this muscle (Fig. 16). Such an explanation for the results of these clinical studies suggest a dose-dependent diffusion phenomenon for the sternocleidomastoid muscle.

Efficacy in the treatment of adult-onset spasmodic torticollis may also depend on diffusion of the biological effects of the toxin from the injection sites. As this disease is definitely associated with multiple muscle group involvement in remote areas of the neck, injection of the entire complex of muscles involved with the posture disfigurement is necessary to achieve the most beneficial result.

Table 3 Response Rates, Comparing Multiple-Point and Single-Point Injections

	Single-Point	Multiple-Point	Mean dose, favorable response (IU)	Mean dose, no response (IU)
Pain	15/31	27/31[a]	165.7	147[b]
Posture deformity	13/42	33/44[a]	162.7	148.3[b]
Range of motion	15/39	33/44[a]	156.4	144.3[b]
Activity	13/39	29/38[a]	187.9	145.6[c]
Hypertrophy	27/39	34/44	161.4	154.6[b]
Tremor	4/17	9/17	163.5	146.9[b]

[a] $p < 0.002$, chi-square.
[b] Not statistically significant (Wilcoxon's test).
[c] $p = 0.04$.

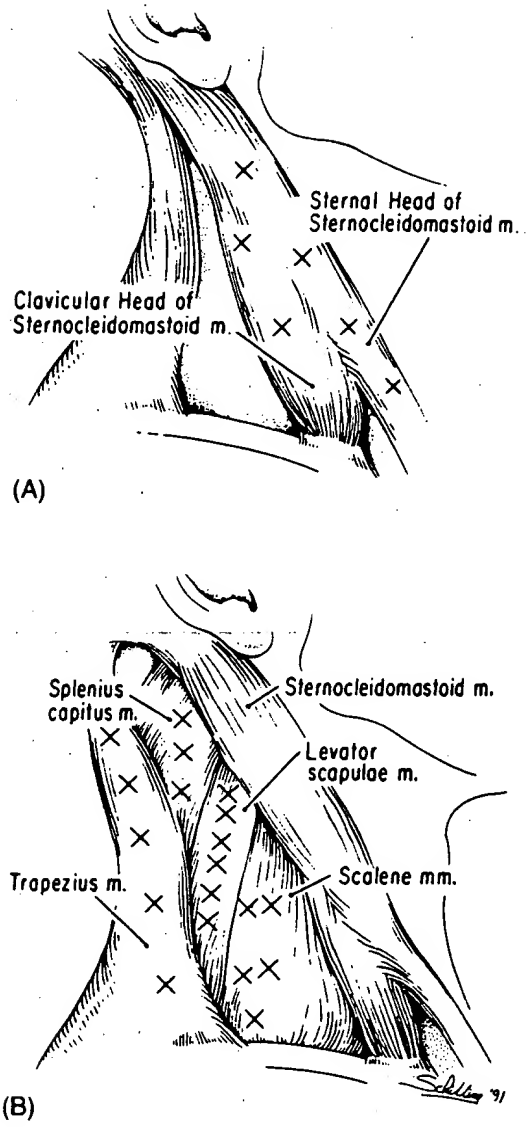
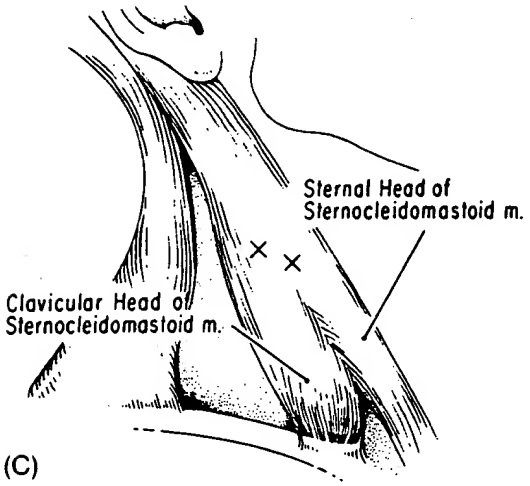


Figure 17 Efficacy and injection technique in the treatment of spasmodic torticollis. In clinical studies, the method of administration is as important to the beneficial results as is the dose of botulinum toxin administered. Figure 17 depicts two types of injection strategy to large dystonic anterior and posterior cervical muscles. The strategy of multiple-point injection per muscle (A,B) produces a superior clinical benefit compared with single injection points per muscle (C,D) or injection points just along a single area. An interpretation of these clinical results is that the multiple-point injection strategy allows a more homogeneous diffusion of the biologic effect of the toxin in the targeted muscles (Table 3).

Given the superior clinical results obtained with multiple-point injections in treating blepharospasm, a study was designed to test multiple-point versus single-point injection per muscle for the treatment of torticollis. By typical efficacy criteria for treatment of spasmodic torticollis—that is, effectiveness with respect to pain, posture deformity, range of motion of the cervical spine, hypertrophy, and activity limitation—better clinical results clearly were achieved in the group receiving multiple-point injections (Table 3, Figure 17). There was no significant difference in the total dose given to each of these



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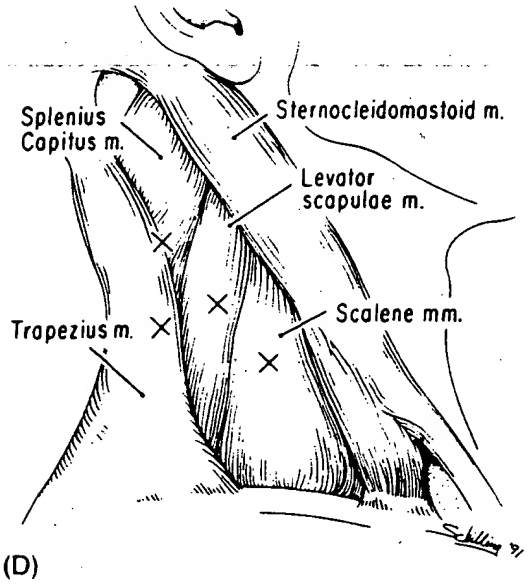

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groups. These findings suggested that the technique of administration within individual large muscles was important to efficacy. It is unknown whether the innervation zone in these muscles is diffusely spread throughout the muscle rather than focally distributed. However, the results of the clinical study tend to suggest a diffuse innervation of most muscles involved with the syndrome. Two of us (G. E. Borodic, R. J. Ferrante) have recently shown that the albino rabbit longissimus dorsi, a very large paraspinal muscle, contains a diffuse innervation zone with periodic bands of NMJs approximately distributed at each spinal nerve root level.

A Depiction of the Diffusion Denervation Field in a Patient

An excellent example of the denervation field produced by a point injection of BTX is the regional depression of the vertical furrowing lines produced by the contraction of the corrugator muscles. These lines are associated with aging. An application demonstrating the denervation field is direct injection of the frontalis muscle in patients with expres-

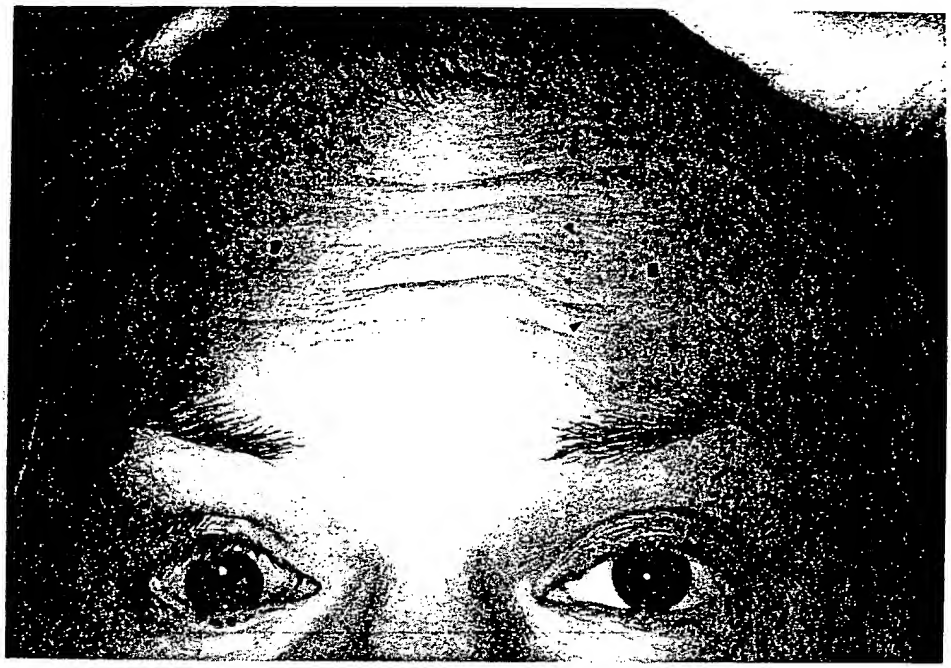


Figure 18 Depiction of denervation field in a patient. The forehead creases are generated by attachments of the frontalis muscle to the skin dermis. This patient was injected along the forehead. Note that the creases are blunted over a circular area after a point injection of botulinum toxin. The blunting of these creases indicates the field of effect of botulinum toxin in this clinical situation.

sionistic overcorrection after ptosis surgery by frontalis sling procedure (65). For instance, the patient shown in Fig. 18 had undergone a frontalis sling to correct total ptosis, using a tendon graft taken from her leg. During the high-amplitude facial movements naturally occurring with emotional expression, the upper lid would become overcorrected. The contractility of this patient's frontalis muscle was reduced on both sides, for symmetry, with point injections of BTX (Fig. 18). This resulted in the blunting of the transverse creases of the forehead over a circular area. The transverse creases are generally produced by the insertion of the frontalis muscle into the dermis of the forehead skin. With high-amplitude contractions and high tone in the muscles, these creases are accentuated. The circular area of blunting of creases indicates decreased tone in the frontalis muscle over a defined region within this muscle. Of note, this patient was injected with 10 IU to each location, and each injection has produced a denervation field of approximately 15 mm radius.

The denervation field is a phenomenon that may vary within various muscle fiber arrangements, after repetitive injections, and with different preparations of the toxin. Further evaluations with respect to these variables are currently under way.

Histologic Determination of the Denervation Field and Botulinum Toxin Activity at Therapeutic Doses

Muscle fiber size, fiber size variability, and AChE staining characteristics were used to assess muscle fiber response and diffusion of biologic activity from a point injection within a long muscle, in an attempt to further define the denervation field scientifically.

The albino rabbit longissimus dorsi muscle was chosen because of its length, generally parallel fiber orientation, diffuse innervation zone, and easy accessibility for muscle biopsy and injection. Subsequent to these studies, a diffuse periodic distribution of NMJs evenly down the length of this muscle was found that further validates this model. Longissimus dorsi muscles in 2- to 3-kg albino rabbits were injected at a point along the middorsal spine (40). At the injection point, a tattoo was applied over the skin and muscle with India ink. In addition to the tattoo, the injection point was anatomically placed at the dorsal eminence of the right levator scapulae to insure reproducible localization of the injection site even if the tattoo faded or tissue plane sliding made identification of the injection point difficult.

Botulinum toxin type A (Oculinum; Oculinum Inc.) was diluted at various concentrations used for clinical study. Control injections were made with 0.9% sodium chloride diluent.

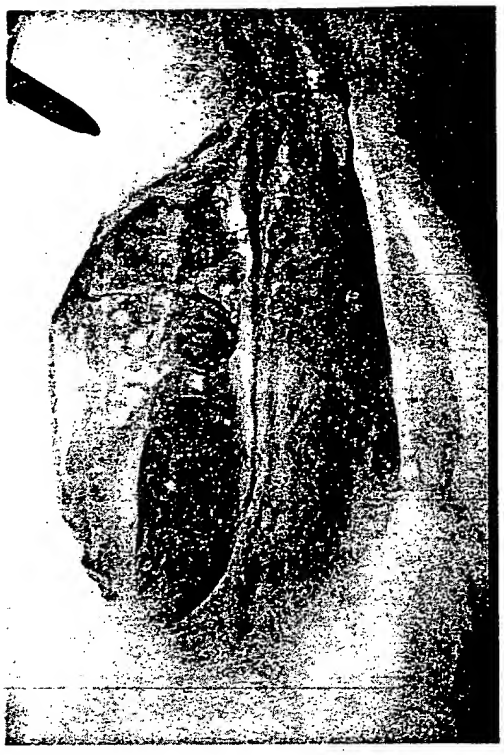
After injection, 5 weeks were allowed to elapse to provide an adequate interval for optimum muscle fiber atrophy and AChE spread (40). Dissection was made over the dorsal spine, removing the latissimus dorsi muscle and exposing the longissimus dorsi muscle down its entire length to the caudal end of the lumbar spine. Biopsy samples were taken at 15-mm intervals and frozen with liquid nitrogen (Fig. 19A). Statistical variations in fiber diameter were compared with F ratio analysis. Fiber diameter and diameter variance analysis were done in each specimen beginning at the injection site and at 15-mm intervals to 45 mm from the injection point.

The intensity of AChE staining was estimated using reference photographs representing gradations of spread and intensity of staining (Fig. 19 B–E). These gradations were rated 0–4. The spread and intensity of AChE staining activity for each biopsy was matched to the closest reference photograph. Four biopsies were developed from each muscle, including controls, and two or three animals were used for each dose determination in the histochemical analysis as well as the analysis of fiber diameter variability and size.

The muscle fiber average diameter was determined from summation of counts on four biopsies taken at 15-mm intervals over a linear distance of 4.5 cm from the injection site. The average muscle fiber diameter through the entire muscle (four biopsies) appeared to correlate to the dose of BTX administered. The average muscle fiber diameter after a 10-IU injection was 26.7 μm ($s = 14.8$) ($n = 1600$), at 5 IU, 31.7 μm ($s = 14.6$) ($n = 1600$), at 2.5 IU, 30.4 μm ($s = 14.0$) ($n = 1600$), and at 1 IU, 30.7 μm ($s = 11.1$) ($n = 2400$). Control fiber diameter was 35.4 μm ($s = 9.2$) ($n = 1600$).

Fiber size variability also correlated directly with the dose administered at the injection site and through the entire muscle (Fig. 20).

The field of biologic activity within the injected muscle was assessed morphologically by measuring fiber size variability and histochemically by means of AChE staining characteristic. The diffusion of biologic activity within the injected muscle correlated with the dose administered. Fiber size variability at 1 IU became insignificant in comparison with controls at 15 mm from the injection point (F ratio <1.4 based on 200 fiber counts per specimen). At 2.5, 5, and 10 IU the biologic effect reflected by fiber size variability was sustained throughout a 45-mm length along the muscle strip (Fig. 21). Fiber size variation was significantly different from controls at all higher doses down the entire muscle strip (F ratio > 1.4). Spread and intensity of AChE staining confirmed that the biological effect substantially diminished at 15 mm for the 1-IU dose. The AChE activity suggested that higher doses (2.5–10 IU) produced a biological effect throughout the 45-mm length of the muscle strip (Fig. 22).



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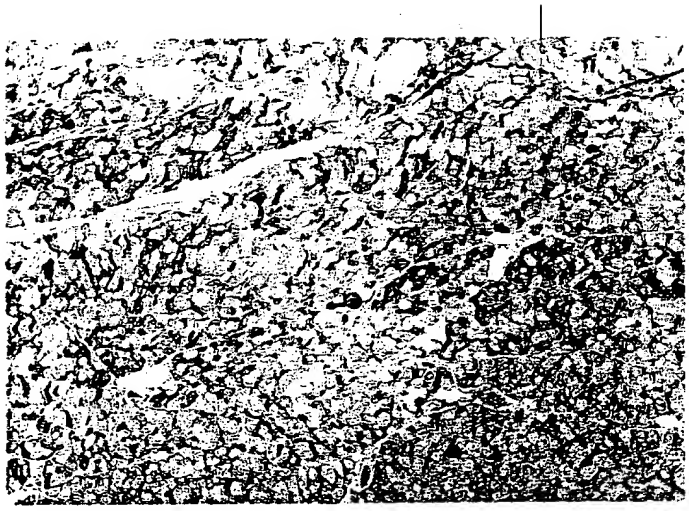
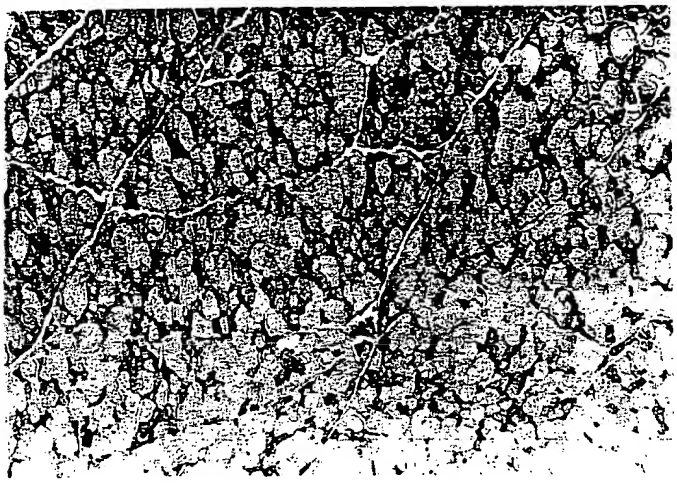


(B)

Figure 19 Animal model for quantization of botulinum toxin diffusion. (A) Animal model for evaluating diffusion of botulinum toxin down longissimus dorsi muscle involves taking multiple biopsies along this muscle. The contralateral muscle is also used for assessment of extramuscular spread. Other contiguous muscles can also be used. (B–E) Diffusion down the longissimus dorsi muscle can be monitored using the acetylcholinesterase staining characteristic. Note that at the injection site after 1.25 IU of botulinum toxin, there is diffuse spread of cholinesterase. Over 45 mm, there is gradual decrease in cholinesterase spreading until the stain become concentrated only at the neuromuscular junctions. The photographs used in this illustration provided reference standards by which tissue at varying distances were used to evaluate diffusion of biologic effect at varying doses (see Figs. 1,2,20,21,22).



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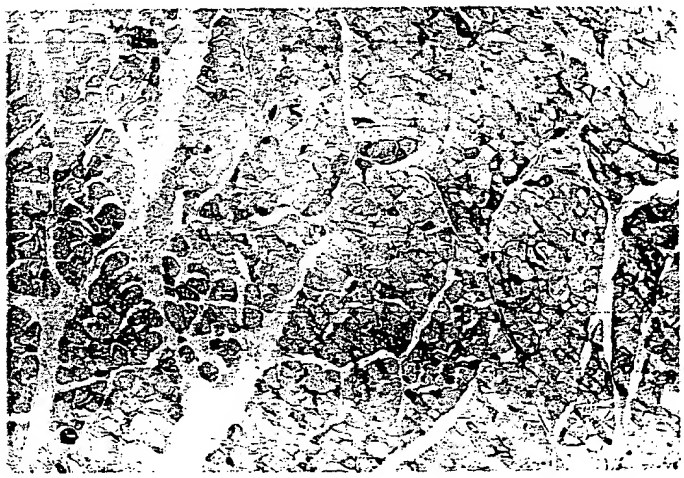

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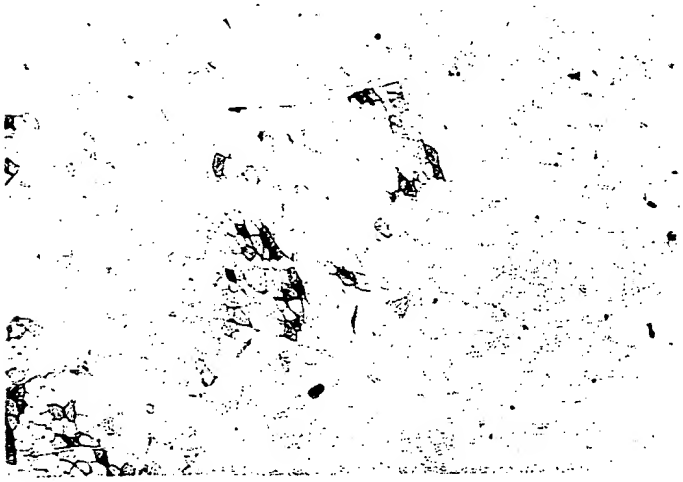

(E)

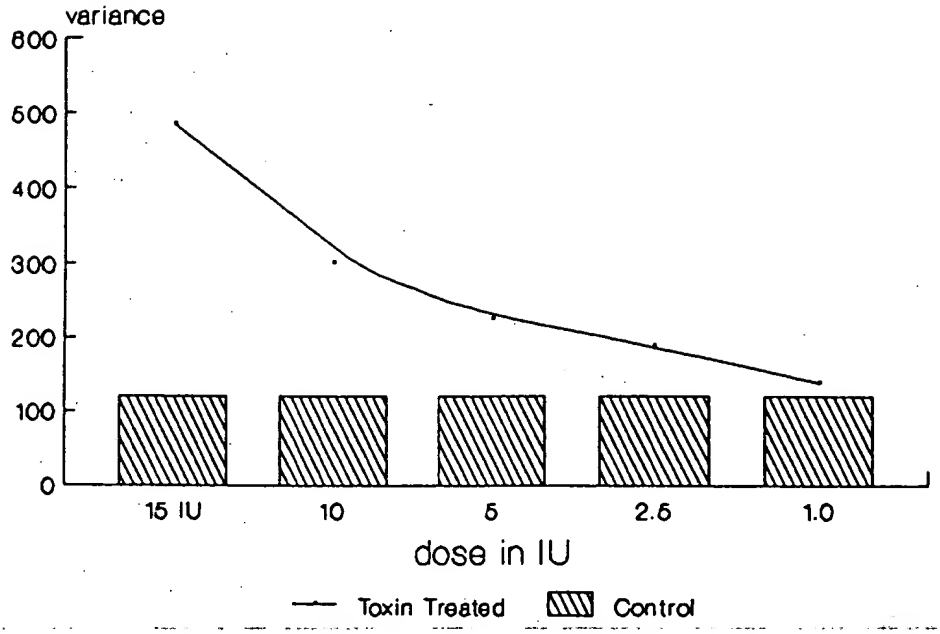

Figure 20 Dose-dependent muscle fiber responses. Fiber size variability correlated directly with the dose at the injection site, as did the average fiber diameter throughout the muscle.

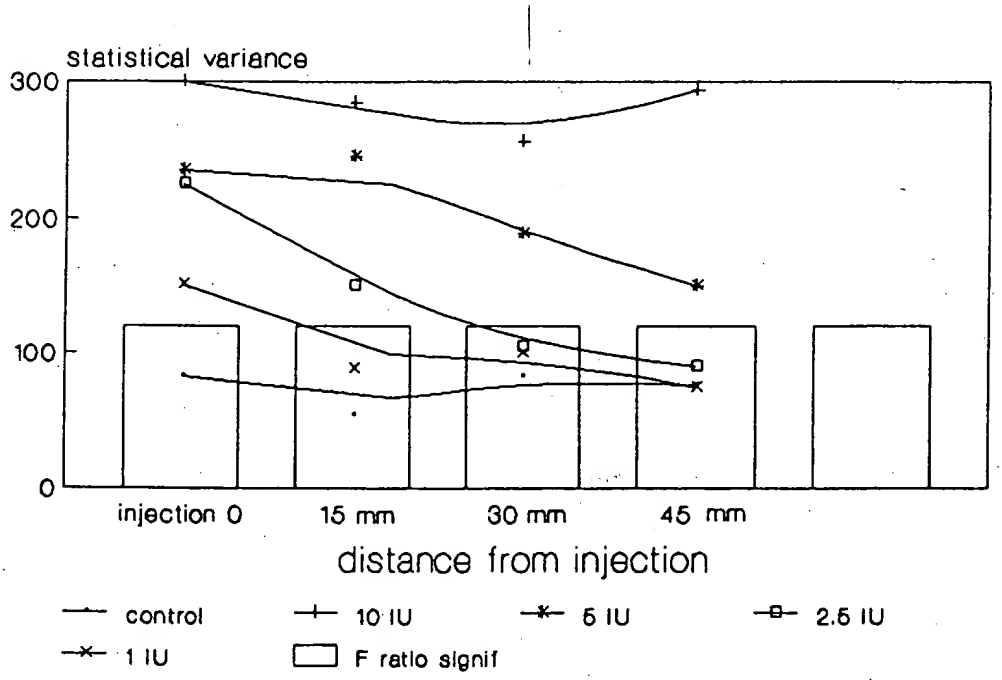

Figure 21 Dose-dependent diffusion. The biological effect within longissimus dorsi muscle appears to be dose-related. The larger the dose the more homogeneous the effect is throughout the muscle strip evaluated. Fiber variations were compared using F ratios.

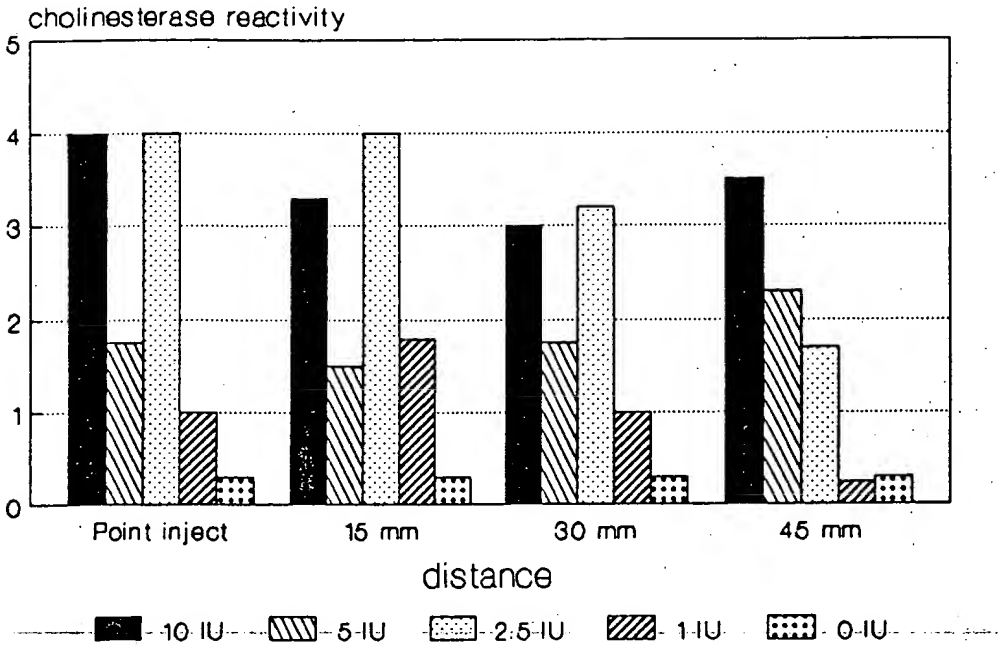

Figure 22 Dose-dependent diffusion: Acetylcholinesterase staining characteristic suggested that higher dose produced a biological effect throughout the entire strip, whereas the smaller dose produced a gradient down the length of the strip studied.

In order to assess extramuscular diffusion properties of BTX-A, fiber diameter variations and fiber diameter size were determined on the contralateral longissimus dorsi muscle at 45 mm from the injection site at each dose. Fiber size variation was significantly greater in the injected muscle at 45 mm than at an extramuscular site 45 mm from the point of injection for 10 IU ($F = 2.5, p < 0.01$) and 5 IU ($F = 1.7, p < 0.01$). For 2.5 IU and 1 IU the differences in fiber size variation between the intramuscular and extramuscular sites were not significant. These data indicated that linear spread of biologic effect may be greater within the injected muscle than in a remote muscle at an equivalent distance from the point of injection of BTX, although the biologic effect did spread to contiguous muscles when larger doses were used.

The AChE activity and the fiber variability pattern confirmed the presence of a dose-dependent field of action for BTX-A.

Diffusion of toxin away from targeted muscles ("toxin jump") appears to cause complications (40). It is therefore useful to attempt to quantify diffusion of biologic activity from a point injection at various doses used in clinical practice. The findings indicate that the degree of fiber atrophy and fiber size variability as well as intensity of AChE staining at the point of injection are directly related to the dose administered. Furthermore, the diffusion of biologic effects from the point of injection within the longissimus dorsi muscle is dose-dependent. Animals given 2.5–10 IU showed substantial diffusion of the toxin's biological effects over a linear distance of 45 mm within this individual large muscle. In contrast, animals given 1 IU demonstrated a graduated biological effect inversely related to the distance from the point injection. There appeared to be collapse of biological effect as indicated by fiber size variation and AChE staining

characteristics between 15 and 30 mm from the point of injection of 1 IU within the injected muscle.

The biological effect in contiguous muscles was evaluated in the longissimus dorsi muscle contralateral to the injection. At lower doses, the toxin's activity did not spread to 45 mm on a muscle remote from the injected muscle. In a previous study (40), the biological activity of BTX has been shown to cross fascial planes and cause histochemical changes in noninjected muscles within lesser distances (<2 cm.). The explanation for the extension of biological effect to a greater distance within the injected longissimus muscle than in extramuscular locations may be that the toxin's activity diffuses to a greater degree within an individual muscle. Alternatively, the linear distance over which chemodenervation occurs may be exaggerated in muscles with very long parallel muscle fibers, such as the longissimus dorsi. At higher doses there appeared to be dissemination of biological activity down the contralateral muscle, although not to the same degree as in the injected muscle.

Containment of biological activity within a targeted area of the body is a desirable goal for BTX injection therapy, and these data offer insight into the intensity and diffusion of biological activity within the injected muscle and at muscles remote from the injection site. Diffusion of biological activity within the muscle appears to be a function of dose and can be graduated. The denervation field can be defined as a linear distance from the point of injection over which BTX causes a denervation effect. The degree of denervation as indicated by fiber size atrophy and fiber size variation is at any distance from the point of infection a function of dose. The size of the denervation field is also a function of dose, as indicated by the homogeneous effects the larger injection doses produced down the long muscle strip. As denervation field size and degree of neurogenic fiber atrophy are both dose-dependent, larger doses of BTX can be expected to produce more weakness and fiber atrophy but with greater spread of the toxin from the injection sites. As complications in clinical practice are often related to undesirable toxin spread, the field of denervation must be considered in the clinical use of BTX.

Since, in clinical practice, muscle atrophy has been noted to occur qualitatively after injection on gross inspection, it is of interest to quantitate the degree of muscle fiber atrophy in this experimental model. From cross-sectional muscle fiber diameter changes after BTX injection, it appears that fiber atrophy of 25% was possible. This analysis is consistent with clinical observations, particularly in the sternocleidomastoid muscles of patients treated for torticollis.

Because the diffusion of biological activity away from a point of injection is dose-related and measurable, it may be possible to calculate in clinical protocols reasonable diffusion fields from a site of a given dose of BTX. Diffusion fields can be established within injected muscles, within contiguous muscles, and within muscles of various fiber orientations. The results of this study underscore the importance of the clinician's being knowledgeable about the anatomical distances between important muscles within the area being injected as well as the action of muscle groups in which BTX is administered. Such information may provide a scientific approach to determining distances between injection sites at various doses. Furthermore, the minimum dose necessary to produce a homogeneous denervation effect down a long muscle would be ideal if just that muscle were being targeted for injection. Doses in excess of the minimum dose for homogeneous denervation would be more prone to spread outside the fascial planes of the muscle and into contiguous muscle groups, potentially causing complications.

Sensitization after Repetitive Botulinum Toxin Injections

According to the literature (66–68), the incidence of sensitization appears to be approximately 3–5% over several years of repeated injections for cervical dystonia (torticollis), and sensitization is found in approximately 30% of patients who lose beneficial response to therapy (67). Sensitization has occurred with both Botox and Dysport. When present, neutralizing antibodies have generally rendered the therapy ineffective.

Previous authors, however, have not demonstrated antibody formation with lower-dose application for the treatment of blepharospasm and Meige syndrome and have argued that at the doses typically used for these indications, it is unlikely that antibodies can form (69,70). In contrast to these reports, the following case demonstrates that antibody-mediated resistance can be associated with treatment failure for low-dose applications such as treatment of blepharospasm.

Case Report

A 57-year-old magazine managing editor presented with visual loss secondary to blepharospasm. The problem gradually developed over several years and became disabling particularly during driving and conversation. Shortly after the onset of involuntary blepharospasm, involuntary lower facial grimacing was noted. The patient's son had developed involuntary hand tremor at the age of 19 years.

Initial therapy included injection of 30 IU (total dose) of BTX-A. There was minimal improvement. Another injection of 60 IU to each eye, was administered with improvement in symptoms. Repeated injections at 3-month intervals involved doses ranging from 50 to 100 IU. After each injection there was some subjective improvement and decreased orbicularis contractility on physical examination. After eight beneficial injections, no benefit was achieved with an additional six injections at 3-month intervals. Orbicularis muscle contractility was not weakened with subsequent injections.

A limited therapeutic myectomy procedure demonstrated no evidence of fiber atrophy or spread of AChE staining activity from the NMJ on the sarcolemma 4 weeks after the last injection (Fig. 23). Figure 2 shows a typical AChE staining response in orbicularis oculi muscle after 5 weeks. A supramaximal quantity of BTX (200 IU) was injected into the patient's eyelids and produced no benefit or orbicularis weakness. A serum specimen was obtained for mouse antibody bioassay.

The patient's serum was tested for neutralizing BTX antibodies. Two samples containing patient plasma were tested, one with BTX-A, and the other with BTX-B. One milliliter of patient plasma was mixed with 1 ml of buffer (30 mM phosphate buffer, pH 6.2, containing 0.2% human serum albumin) and 25.7 IU of either BTX-A or BTX-B. Two control samples with buffer only plus toxin were simultaneously run for each of the toxin serotypes. These mixtures were allowed to incubate at room temperature for 1 hour before analysis for biological activity. A mouse bioassay was used to determine the levels of BTX in any sample. A 0.2-ml (2.57 IU toxin) aliquot of each of the mixtures was injected into five mice. Following injection the animals were monitored for 5 days, and the total deaths in each group were determined.

To provide an estimate of the amount of antitoxin present in the patient plasma, a series of dilutions of plasma were examined. Probit analysis of titration data is shown in Table 4 and indicates that the LD_{50} was at the 1:3.96 dilution. At this dilution there was enough antitoxin present in the diluted plasma to neutralize 1.57 IU of BTX-A. On the basis of these calculations, 1 ml of this patient's plasma contained enough antitoxin to neutralize

Figure 23 Lack of tissue response in a patient with circulating anti–botulinum A toxin antibody: This orbicularis oculi biopsy was stained for acetylcholinesterase in a region injected with botulinum toxin 5 weeks before the procedure. Note that the acetylcholinesterase activity remains confined to a neuromuscular junction and is not diffusely staining (see Figs. 2,19,22). This patient subsequently was found to have developed botulinum A toxin antibodies (see Table 4).

31.1 IU of BTX-A. Expressed in terms of international units of antitoxin, this is 0.0003 IU/ml of plasma.

Titration of the amount of antibody in plasma was accomplished by examining the neutralizing properties of seven dilutions of patient plasma: 1:1, 1:2, 1:4, 1:8, and 1:16. One milliliter of each dilution was incubated for 1 hr with an equal volume of buffer containing 25.7 IU of BTX-A. Five mice were injected with 0.2 ml of each test dilution. The mice were monitored for 5 days and the mortality with each dilution was determined. The dilution at which 50% death occurred was determined by probit analysis. At the

Table 4 Toxin vs Serum or Buffer

	Percent Mortality	
Conditions	Test 1	Test 2
BTX-A + serum	0	0
BTX-A + buffer	100	80
BTX-B + serum	100	100
BTX-B + buffer	80	100

LD_{50}, there was sufficient antitoxin present to neutralize 3 IU of BTX-A, and 1 IU remained. Estimations of the antibody titers in the patient plasma were determined on this basis.

Presented in Table 4 are the results from two tests in which patient plasma was diluted 1:1 with buffer containing 20 IU of either BTX-A or BTX-B. These results show that patient plasma inactivated only samples containing BTX-A, whereas samples containing BTX-B were unaffected. The controls indicate that these results cannot be accounted for on the basis of low BTX activity. This test indicates only that there was at least enough antitoxin present in 1 ml of patient plasma to neutralize 4 IU of BTX-A.

This case report demonstrates immunologically mediated resistance causing a poor response after repeated injections in a patient who received relatively small amounts of therapeutic BTX. The presence of neutralizing antibody specific for BTX-A was demonstrated. Furthermore, tissue resistance to BTX injection was demonstrated with muscle biopsy using AChE staining. The true incidence of circulating antibodies in patients with various diseases treated with BTX is still an open question. There is also a need for a conventional assay to measure circulating antibodies.

Biological Activity of Preparations and Immunological Considerations

When BTX is prepared from ammonium sulfate crystals with other inert proteins, the preparation is known as the botulinum toxin protein complex (41). Botox is a BTX protein complex. Further protein purification can be used, removing inert proteins from the BTX protein complex and yielding higher grade of neurotoxin per quantity of protein. An index useful in evaluating the relative purity of a biologically active protein preparation is specific activity. Specific activity is defined as the ratio of biologic activity (IU using the standard mouse assay) to the quantity of protein present in the preparation as determined photometrically (71,72). The specific activity of the first preparation used in clinical studies was 2.5 IU/ng protein in the preparation. Some initial efforts have been made to ensure that reasonably pure protein preparations are used clinically. The batches used to prepare the pharmaceutical material are required to demonstrate greater than 1,000,000 LD_{50}/ml before dilution and lyophilization. However, the specific activity of the final preparation can be influenced by each step of the pharmacological preparation process. The potential significance of specific activity in clinical practice is unknown, but it may have a bearing on the immunogenicity of the final therapeutic agent. The antigenicity of BTX preparations is clearly an issue requiring further careful studies.

Toxic Dose of Botulinum Toxin in Man

One international unit is the LD_{50} dose for a 20- to 30-g white mouse. Over the past decade this unitage has been adapted to clinical application for the treatment of regional movement disorders. It has been estimated that approximately 3000 IU are needed to produce BTX intoxication in man (73).

SUMMARY

1. Diffusion of therapeutic botulinum toxin from points of intramuscular injection appears to be a dose-dependent phenomenon. Intensity of the denervative effect is also dose-dependent.

2. Diffusion of the denervative effect outside the muscles targeted for injection ("toxin jump") is responsible for side effects associated with the clinical use of botulinum toxin.
3. Limiting the dose of BTX in critical anatomical areas can be helpful in preventing complications (e.g., limiting the sternocleidomastoid muscle dose to prevent dysphagia in patients treated for spasmodic torticollis patients).
4. Multiple-point injections within targeted muscles have produced the most desirable clinical effects in patients with blepharospasm and adult-onset spasmodic torticollis. This injection approach may be more beneficial because it diffuses the biologic effect of the toxin more evenly throughout the innervation zone of the muscle.
5. Muscle fiber size variability and spread of AChE on muscle fibers are consistent histologic markers for therapeutic BTX effect. A dose-dependent gradient of biological activity can be demonstrated both within muscles injected and within adjacent muscles.
6. Long-term, repetitive treatments with therapeutic injections do not seem to produce permanent denervative effects as assessed through fiber size variation and cholinesterase staining characteristics in human muscle specimens.
7. Sprouting appears to cause changes in terminal axonal projections into muscle fibers, so that more fibers are innervated by a terminal axon.
8. Sensitization to BTX-A may occur after repetitive injections and can be an explanation for poor responses after such injections.

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REFERENCES

1. Scott AB. Botulinum toxin injections to eye muscles to correct strabismus. J Am Ophthalmol Soc 1981;79:734–770.
2. Scott AB, Magoon EH, McNeer KW, Stager DR. Botulinum treatment of strabismus in children. Trans Am Ophthalmol Soc 1990;87:174–180; discussion 180–184.
3. Scott AB. Strabismus injection treatment. In: NIH consensus development conference on clinical use of botulinum toxin, November 12–14, 1990. 117–118.
4. Scott AB, Kennedy EG, Stubbs HA. Botulinum A toxin injection as a treatment for blepharospasm. Arch Ophthalmol 1985;103:347–350.
5. Borodic GE, Cozzolino D. Blepharospasm and its treatment, with emphasis on the use of botulinum toxin. Plast Reconstr Surg 1989;83:546–554.
6. Borodic GE, Mills L, Joseph M. Botulinum A toxin for the treatment of adult-onset spasmodic torticollis. Plast Reconstr Surg 1991;87:285–289.
7. Borodic GE, Cozzolino D, Townsend DJ. Dose-response relationships in patients treated with botulinum toxin for more than three years (abstr). Sixth International Meeting of the Benign Essential Blepharospasm Research Foundation: 1988, August 25–27; Cambridge, MA. Ear Nose Throat J 1988;67:914.
8. Jankovic J, Orman J. Botulinum toxin for cranial cervical dystonia: a double blind placebo controlled study. Neurology 1987;37:616–623.

9. Blitzer A, Brin MF, Greene PE, Fahn S. Botulinum toxin injection for the treatment of oromandibular dystonia. Ann Otol Rhinol Largyngol 1989;98:93–97.
10. Fletcher NA, Quinn N. Dystonic syndromes. Curr Opin Neurol Neurosurg 1989;2:330–333.
11. Gelb DJ, Lowenstein DH, Arminoff MJ. Controlled trial of botulinum toxin injections in the treatment of spasmodic torticollis. Neurology 1989;39:80–84.
12. Borodic GE, Pearce LB, Joseph M. Cranial cervical dystonias, multiple vs single injection strategies. A clinical study. Head Neck 1992;14:33–37.
13. Kao I, Drachman D, Price DL. Botulinum toxin: mechanism of presynaptic blockade. Science 1976;193:1256.
14. Tse CK, Dolly JO, Hambleton P, Wray D, Melling J. Preparation and characterization of homogeneous neurotoxin type A from Clostridium botulinum. Its inhibitory action on neuronal release of acetylcholine in the absence and presence of bungarotoxin. Eur J Biochem 1982;122:493–500.
15. Evans DM, Williams RS, Stone CC, Hambleton P, Melling J, Dolly JO. Botulinum neurotoxin type B: its purification, radioiodination and interaction with rat brain synaptosomal membranes. Eur J Biochem 1986;154:409–416.
16. Dasgupta BR. Structure and biological activity of botulinum neurotoxin. J Physiol (Paris) 1990;84:220–228.
17. Singh BR, Dasgupta BR. Molecular topography and secondary structure comparisons of botulinum neurotoxin types A, B and E. Mol Cell Biochem 1989;86:87–95.
18. Sakaguchi G, Ohishi I, Kozaki S. Purification and oral toxicities of Clostridium botulinum progenitor toxins. In: Biomedical aspects of botulinum toxin. Academic Press, 1981:21–33.
19. Black JD, Dolly JO. Selective location of acceptors for botulinum neurotoxin A in the central and peripheral nervous systems. J Cell Biol 1986;103:521–534.
20. Williams RS, Tse CK, Dolly JO, Hambelton P, Melling J. Radioiodination of botulinum neurotoxin type A with retention of biologic activity and its binding to brain synaptosomes. Eur J Biochem 1983;131:437–445.
21. Molgo J, Comella JX, Angaut-Petit D, Pecot-Dechavassine M, Tabit N, Faille L, Mallart A, Thesleff S. Presynaptic actions of botulinal neurotoxins at the vertebrate neuromuscular junctions. J Physiol (Paris) 1990;84:152–166.
22. Ashton AC, Dolly JO. Microtubule-dissociating drugs and A23187 reveal differences in the inhibition of synaptosomal transmitter release by botulinum neurotoxins types A and B. J Neurochem 1991;56:827–835.
23. Gansel M, Penner R, Dreyer F. Distinct sites of action of clostridial neurotoxins revealed by double-poisoning of mouse motor nerver terminals. Eur J Physiol 1987;409:533–539.
24. Schmitt A, Dreyer F, John C. At least three sequential steps are involved in tetanus toxin–induced block of neuromuscular transmission. Naunyn-Schmiedebergs Arch Pharmacol 1981;317:326–330.
25. Simpson LL. The origin, structure, and pharmacological activity of botulinum toxin. Pharmacol Rev 1981;33:155–188.
26. DasGupta BR, ed. Proceedings of the International Conference on Botulinum and Tetanus Neurotoxins. Madison, Wisconsin, May 1992.
27. Duchen LW. Changes in motor innervation and cholinesterase localization induced by botulinum toxin in skeletal muscle of mouse: differences between fast and slow muscles. J Neurol Neurosurg Psychiat 1970;33:40–54.
28. Duchen LW. Histologic differences between soleus and gastrocnemius muscles in the mouse after local injection of botulinum toxin. J Physiol (Lond) 1969;204:17–18.
29. Aldersen K, Holds JB, Andersen RL. Botulinum induced alteration of nerve muscle interactions in human orbicularis oculi following treatment for blepharospasm. Neurology 1991;41:1800–1805.
30. Schuetze SM, Role LW. Developmental regulation of nicotinic acetylcholine receptors. Annu Rev Neurosci 1987;10:403–457.

31. Fertuke HC, Salpeter MM. Localization of acetylcholine receptors by I 125a bungarotoxin binding at mouse neuromuscular junction. Proc Natl Acad Sci USA 1974;71:1376–1378.
32. Bevan S, Steinbach JH. The distribution of bungarotoxin binding sites on mammalian skeletal muscle developing in vivo. J Physiol 1977;267:195–215.
33. Fambrough DM. Acetylcholine receptor: revised estimates of extrajunctional receptor density in denervated rat diaphragm. J Gen Physiol 1974;64:468–472.
34. Miledi R. The acetylcholine sensitivity of from muscle fibers after complete and partial denervation. J Physiol 1960;151:1–23.
35. Goldman D, Staple J. Spatial and temporal expression of acetylcholine receptor RNAs in innervated and denervated rat soleus muscle. Neuron 1989;3:219–228.
36. Pestronk A, Drachman DB, Griffin JW. Effect of botulinum toxin on trophic regulation of acetylcholine receptors. Nature 1976;264:787–789.
37. Yee WC, Pestronk A. Mechanisms of postsynaptic plasticity: remodeling of the junctional acetylcholine receptor cluster induced by motor nerve terminal outgrowth. J Neurosci 1987;7:2019–2024.
38. Angaut-Petit D, Molgo J, Comella JX, Faille L, Tabti N. Terminal sprouting in mouse neuromuscular junctions poisoned with botulinum type A toxin: morphological and electrophysiological features. Neuroscience 1990;37:799–808.
39. Borodic GE, Ferrante R. Histologic effects of repeated botulinum toxin over many years in human orbicularis oculi muscle. J Clin Neuro-Ophthalmol 1992;12:121–127.
40. Borodic GE, Joseph M, Fay L, Cozzolino D, Ferrante R. Botulinum A toxin for the treatment of spasmodic torticollis. Dysphagia and regional toxin spread. Head Neck 1990;12:392–398.
41. Borodic GE, Pearce LB, Johnson EJ, Schantz E. Clinical and scientific aspects of botulinum toxin. Ophthalmol Clin N Am 1991;43:491–503.
42. Jankovic J, Brin M. Therapeutic uses of botulinum toxin. N Engl J Med 1991;324:1194.
43. Kao I, Drachman DB. Motor nerve sprouting and acetylcholine receptors. Science 1978;193:1256.
44. Duchen LW, Stritch SJ. The effects of botulinum toxin on the pattern of innervation of skeletal muscle in the mouse. Q J Exp Physiol 1968;53:84–89.
45. Gillum WN, Anderson RL. Blepharospasm surgery: an anatomical approach. Arch Ophthalmol 1981;99:1056–1062.
46. Holds JB, Alderson K, Fogg SG, Anderson RL. Terminal nerve and motor end plate changes in human orbicularis muscle following botulinum A toxin injection. Invest Ophthalmol Vis Sci 1990;31:178–181.
47. Wohlfart G. Collateral sprouts from residual motor nerves in amyotrophic lateral sclerosis. Neurology 1957;7:124–134.
48. Alderson K, Yee WC, Pestronk A. Reorganization of intrinsic components in the distal motor axon during outgrowth. J Neurocytol 1989;18:541–552.
49. Duchen LW, Tonge DA. The effects of implantation of an extra nerve on axonal sprouting usually induced by botulinum toxin in skeletal muscle of the mouse. J Anat 1977;124: 205–215.
50. Edds MV. Collateral regeneration of residual motor axons in partially denervated muscles. J Exp Zool 1950;113:517–551.
51. Hoffman H. Local reinnervation in partially denervated muscle. A histophysiological study. Aust J Exp Biol 1950;28:383–397.
52. Hopkins WG, Brown MC, Keynes RJ. Nerve growth from nodes of Ranvier in inactive animals. Brain Res 1981;222:125–128.
53. Brown MC, Holland RL, Hopkins WG. Motor nerve sprouting. Annu Rev Neurosci 1981;4:17–42.
54. Harris CP, Alderson K, Nebeker J, Holds JB, Anderson RL. Histology of human orbicularis muscle treated with botulinum toxin. Arch Ophthalmol 1991;109:393–395.
55. Spencer RF, McNeer KW. Botulinum toxin paralysis of adult monkey extraocular muscles. Arch Ophthalmol 1987;105:1703–1711.

56. Jankovic J, Ford J. Blepharospasm and oro-facial dystonia. Pharmacologic findings in 100 patients. Ann Neurol 1979;36:635.
57. Frueh BR, Callahan A, Dortzbach RR et al. The effects of differential section of the seventh nerve on patients with blepharospasm. Trans Am Acad Ophthalmol Otolaryngol 1976;81:595.
58. Callahan A. Surgical correction of intractable blepharospasm, technical improvement. Am J Ophthalmol 1965;60:788.
59. Janetta PJ, Abbasy M, Maroon JC, Ramos FM, Albin MS. Etiology and differential microsurgical treatment of hemifacial spasm. J Neurosurg 1977;47:321.
60. Borodic GE, Metson R, Townsend D, McKenna M, Pearce LB. Botulinum toxin for aberrant facial nerve regeneration. Dose response relationships. Plast Reconstr Surg 1991;
61. Frueh BR, Nelson CC, Kapustiak JF, Musch DC. The effects of omitting the lower eyelid in blepharospasm treatment. Am J Ophthalmol 1988;106:45–47.
62. Borodic GE, Weigner A, Ferrante R, Young R. Orbicularis oculi innervation zone and implications for botulinum A toxin therapy for blepharospasm. Ophthalm Plast Reconstr Surg 1991;7:54–59.
63. Bertrand C, Molina-Negro P, Martinez SN. Technical aspects of selective peripheral denervation for spasmodic torticollis. Appl Neurophysiol 1982;45:326–330.
64. Bertrand C, Molina-Negro P, Bouvier G, Gorczyca W. Observations and results in 131 cases of spasmodic torticollis after selective denervation. Appl Neurophysiol 1987;50:319–323.
65. Borodic GE. Botulinum A toxin application for expressionistic ptosis overcorrection after frontalis sling procedures. Ophthalm Plast Reconstr Surg 1992;8:137–142.
66. Tsui JK, Wong NLM, Wong E, Calne DB. Production of circulating antibodies to botulinum-A toxin in patients receiving repeated injections for dystonia (abstr). Ann Neurol 1988;23:181.
67. Jankovic J, Schwartz K. Clinical correlates of response to botulinum toxin injections. Arch Neurol 1991;48:1253–1256.
68. Hambleton P, Cohen HE, Palmer BJ, Melling J. Antitoxins and botulinum toxin treatment. Br Med J 1992;304:959–960.
69. Biglan AW, Gonnering R, Lockhart LB, Rabin B. Fuerste FH. Absence of antibody production in patients treated with botulinum A toxin. Am J Ophthalmol 1986;101:232–235.
70. Gonnering RS. Negative antibody response to long-term treatment of facial spasms with botulinum toxin. Am J Ophthalmol 1988;105:313–315.
71. Cardella MA. Botulinum toxoids. In: Lewis KH, Cassel K Jr, eds. Botulism: proceedings of a symposium. PHS Publication No. 999-FPI. Washington, D.C., Government Printing Office, 1964:113–130.
72. Hatheway CH, Snyder JD, Seals JE, Edell TA, Lewis GE Jr. Antitoxin levels in botulism patients with trivalent equine botulism antitoxin to toxin types A,B, and E. J Infect Dis 1984;150:407–412.
73. Scott AB, Suzuki D. Systemic toxicity of botulinum toxin by intramuscular injection in the monkey. Mov Disord 1988;3:333–335.